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EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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COMPARATIVE PATHOLOGY OF SOUTH AFRICAN JAGZIEKTE AND MONTANA PROGRESSIVE PNEUMONIA OF SHEEP.

By E. V. COWDRY and HADLEIGH MARSH.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York, and the Montana Livestock Sanitary Board, Helena.)

Plates 16 to 18.

(Received for publication, November 29, 1926.)

Recent studies on a disease of sheep occurring in the northwestern United States, called "progressive pneumonia,"[1,2] appear to have a very definite bearing upon our conception of a South African disease called jagziekte, and *vice versa*. The two conditions have been studied independently, the investigators in each case considering the disease as unique and peculiar to their respective geographical localities. Comparison of publications on the two diseases has brought to our attention the fact that these two chronic pneumonias of sheep, occurring in widely separated parts of the world, are very similar, and apparently are identical in the fundamental characteristics which differentiate them from other described forms of pneumonia. It therefore seems profitable to correlate our knowledge of two such closely related diseases by making a comparative study of the lesions, particularly as in both cases, the diseases cause a considerable loss to the sheep industry, and in neither case has the etiology been definitely established.

Jagziekte has been known in South Africa since 1893. The term is derived from the Dutch *jagt*, to drive, and *ziekte*, a sickness, and is intended to call to mind the fact that the first symptoms appear in animals which are fatigued as a result of having been driven for some distance. The principal lesions are pneumonic and proliferative in character. We have shown the tumor-like proliferations of pulmonary epithelium to several investigators very familiar with cancer. Some

[1] Marsh, H., *J. Am. Vet. Med. Assn.*, 1922–23, lxii, 458.

[2] Marsh, H., *J. Am. Vet. Med. Assn.*, 1923–24, lxiv, 304.

do not hesitate to return a diagnosis of true cancer, while others are more conservative and remark, that, if the same conditions were met with in the human lung, they would classify them as cancerous. No cases have been known to recover. The disease is certainly contagious and is so greatly feared that the farmers immediately kill off all contacts, although they may appear to be entirely healthy.[3]

"Progressive pneumonia" of sheep has been studied in Montana since 1915. Sheep affected with this disease are called "lungers" by the sheepmen. Many sheepmen state that they have known the condition for the last 30 years, but it has apparently increased in recent years and invaded new territory. This is apparently a disease of range sheep as opposed to farm flocks. It is believed that the disease is infectious, and that the infection is probably secondary to mechanical causes. The lesions are similar to those of jagziekte and the prospect of recovery is just as hopeless.

The present study was accordingly commenced in the hope that a detailed comparison of the two conditions might reveal points of correspondence or of divergence, which have hitherto been overlooked, because they have never before been considered together; and, further, that this new information might in turn be of service in unraveling the obscure etiology of both diseases.

Material.

The material upon which this paper is based consists of tissues collected in South Africa from 33 sheep suffering from jagziekte and from 72 control healthy sheep (including some American sheep employed as geographic controls). In addition many clinical cases of jagziekte were observed. The progressive pneumonia material consists of tissues from 46 cases and from 18 supposedly normal sheep. Both histological and bacteriological examinations were made in progressive pneumonia and the symptoms of many typical cases were noted.

OBSERVATIONS.

Before reporting our studies on the comparative pathology of jagziekte and progressive pneumonia, the accompanying table is submitted in order to give a general outline of the two conditions.

Specific reference may be made to one statement in the table; namely, that enclosures in which sheep suffering from jagziekte have been kept constitute sources of infection for incoming healthy sheep, which apparently does not hold

[3] Mitchell, D. T., *3rd and 4th Rep. Director Vet. Education and Research* (Sir Arnold Theiler), Union of South Africa, 1915, 585.

TABLE I.

	Jagziekte	Progressive pneumonia
Epidemiology	1. Average mortality in infected areas 1.6 per cent *per annum*	1. About 2 per cent *per annum* in infected bands
	2. Occurs throughout the year	2. The same
	3. All ages affected, but chiefly at 3 yrs.; never lambs	3. Most of the deaths occur in sheep 4 yrs. old and over; but many clinical cases have been observed in 2 yr. olds, a few in yearlings, and early stages have been seen in lambs on postmortem examination
	4. Occurs in both range sheep and farm flocks, but chiefly the former	4. Occurs in range sheep, but not in farm flocks
	5. High altitude and low humidity	5. The same
	6. High mean annual temperature	6. Lower mean annual temperature
	7. No evidence that the character of water supply and of the grazing are determinative factors	7. The same
	8. Apparently transmitted by contact, since it appears after importation of new sheep	8. The same
	9. Enclosures in which cases of jagziekte have been kept are said to constitute sources of infection for healthy sheep for 10 days or more after the diseased sheep have been removed	9. Not known to be the case
Symptoms	1. Increasing respiratory distress with catarrhal nasal discharge	1. Increasing respiratory distress, with but little nasal discharge in most cases
	2. Extreme emaciation	2. The same
	3. Little or no increase in temperature. Failure to note a temperature reaction may be due to the fact that the sheep are seldom observed clinically in the earliest stages of the disease, which are in fact not detectable	3. The same

TABLE I—*Continued.*

	Jagziekte	Progressive pneumonia
Symptoms—*continued*	4. The disease extends over a period of several mos. At the outset destructive lesions may exist which are not revealed by clinical symptoms	4. The disease extends over a period of several mos. after symptoms appear. The period during which it exists, but cannot be detected clinically, may extend for several mos. or even yrs.
Prognosis	Duration of life may be prolonged by careful treatment, but it is said to be invariably fatal.[3] There is, however, some evidence that animals in the earliest stages may recover[4]	The same
Prophylaxis	Immediate slaughter of all contacts and the abandonment or careful cleaning of quarters occupied by sick sheep	Slaughter of all recognizable cases
Etiology	1. No organisms have thus far been constantly observed in the specific lesions, although bacteria of several kinds are frequently present, usually in association with inhaled dust-like foreign material	1. Two bacteria have been constantly isolated in cultures made from the lungs, one or both of which may be the causative agents[2]
	2. Eosinophil leucocytes, which one would expect in nematode infestations, are not unusually abundant	2. The same
	3. Poor general condition and insufficient food are not of primary importance as predisposing factors	3. The same
	4. It has never been possible to transmit the disease by the inoculation of material of any kind from diseased animals	4. Repeated intratracheal inoculations of a diphtheroid from affected sheep produced small lesions of the same character as the extensive lesions found in naturally diseased animals. One sheep which was inoculated intratracheally with lung tissue developed the disease

TABLE I—*Concluded.*

	Jagziekte	Progressive pneumonia
Etiology—*continued*	5. The intratesticular and intracerebral inoculation of tissue—procedures indicated by the tumor-like appearance of the epithelial growth—have not been attempted	5. The same
Pathology	1. The specific lesions are restricted to the thoracic cavity	1. The same
	2. The primary changes are found in the interalveolar tissue of the lungs. They occur in foci several mm. in diameter and consist of engorgement of the alveolar capillaries and of the interstitial accumulation of macrophages and lymphocytes. Frequently they are associated topographically with deposits of above mentioned foreign material[4]	2. The same, except that this primary interalveolar infiltration seems to be more extensive
	3. Exudation of macrophages and of leucocytes then takes place into the alveoli resulting in a typical chronic catarrhal pneumonia. At the same time there is a marked and very luxuriant tumor-like proliferation of the epithelial cells of the alveoli and bronchioles[5]	3. The same, except that the epithelial proliferation is usually less pronounced and resembles more a benign hyperplasia. The growth is distinctly less invasive and less neoplastic in appearance. Dividing epithelial cells are more abundant, also alveolar casts
	4. There is sometimes a slight tendency toward localized resolution of the pneumonic process, but this is rare and never takes place on a large scale	4. Not observed
	5. The blood supply is reduced and extensive fibrosis takes place. The animals apparently die from asphyxiation through reduction in respiratory area	5. The same

in progressive pneumonia. It is based upon casual observation only and is difficult to prove, because it has been found that jagziekte, in its earliest stages, is not recognizable clinically[3,4] so that supposedly healthy sheep employed in the experiments may not be in reality free from the disease to begin with.

Examination of gross specimens shows that the lesions commence in definite foci and spread thence throughout the pulmonary tissue. In the more central parts of the affected areas the lesions are older, and fibrosis and consolidation have frequently set in. It is possible, therefore, by selecting specimens for histological examination, first remote from the principal lesions and then nearer and nearer to them, to secure a series of preparations which give some idea of the sequence of changes involved. The earliest changes may, we believe, occasionally also be found by the examination of sheep which have as yet shown no clinical signs of the disease, but which come from affected flocks, through a comparison of their tissues with those of sheep known to be free from both diseases.[4] Lungs from cases of jagziekte of this kind show the infiltrative, exudative and slightly proliferative changes which we are about to describe, uncomplicated by the pneumonia and fibrosis characteristic of more advanced stages.

In the plates illustrating the lesions, the photomicrographs on the left are from cases of jagziekte and those on the right from cases of progressive pneumonia. For additional illustrations of the diseases considered separately, reference may be made to earlier publications.[1–5]

The Interalveolar Tissue.

In both diseases the primary changes center in the interalveolar tissue, which is thickened (Figs. 1 and 2). They are identical in quality but seem to be rather more extensive in progressive pneumonia.

The thickenings are caused principally by accumulations of lymphocytes and large mononuclear cells which have been variously called macrophages (Metchnikoff), endothelial leucocytes (Mallory) and polyblasts (Maximow). None of them exhibits signs of mitotic division. Sometimes, in both conditions, the lymphocytes may greatly outnumber the large mononuclears and give rise to nodules

[4] Cowdry, E. V., *J. Exp. Med.*, 1925, xlii, 323.
[5] Cowdry, E. V., *J. Exp. Med.*, 1925, xlii, 335.

recognizable in gross specimens. The nodules are often peribronchial in position. Both of these cells are accompanied by only a few polymorphonuclear leucocytes. Eosinophil leucocytes are not unusually abundant either in the lesions or in the circulating blood.

The exact source of these large mononuclears is difficult to ascertain, although they are probably chiefly of hematogenous origin. No evidence was found that they are produced through the multiplication of the endothelial cells of the alveolar capillaries as claimed by Permar.[6] In some jagziekte preparations they were seen within the capillary lumina in contact with erythrocytes and free from the lining endothelium (foot-note 4, Fig. 9). In all likelihood they may also arise from cells resident in the interalveolar tissue; in other words, from the rhagiocrine cells of Renaut, or the histiocytes of Kiyono (to employ only a few of the available synonyms). That they may be derived from the lymphocytes which are likewise present normally in small numbers in the interalveolar tissue is to be considered as a third possibility in view of Maximow's[7] observation that freshly emigrated lymphocytes are capable of transformation into large phagocytic ameboid cells; that is to say, into the mononuclear cells. The lymphocytes, in turn, may emigrate from the alveolar capillaries, since, although we have no specific information regarding the pulmonary lymphatics of sheep, Miller[8] was unable to find in man any lymphatics in the walls of the air sacs beyond the ductuli alveolares.

The thickenings in jagziekte frequently also contain, in addition to the cells enumerated, deposits of inhalted foreign material which have been already reported,[4] and there is reason to believe that in progressive pneumonia substances of this kind may likewise act as predisposing factors.

With the foreign material in jagziekte, bacteria of several kinds are frequently noted on microscopic examination, and it is probable that cultural methods would bring to light the existence of a varied bacterial flora. Several bacterial invaders are probably also concerned in progressive pneumonia, the bacteriology of which has been much more systematically studied.[2]

The fact that Marsh succeeded in experimentally producing small lesions, like those of typical cases of progressive pneumonia, by the intratracheal inoculation of a diphtheroid isolated from affected sheep

[6] Permar, H. H., *J. Med. Research.*, 1920–21, xlii, 147, Plate IV.
[7] Maximow, A. A. *Physiol. Rev.*, 1924, iv, 533.
[8] Miller, W. S., *Am. Rev. Tuberc.*, 1919–20, iii, 193.

is suggestive, especially in view of some recent experiments by Grumbach,[9] who, by the injection, similarly of a diphtheroid (*"Corynebacillus diphtheroide"*), caused pulmonary lesions in guinea pigs, which he regards as almost identical with those of jagziekte in sheep. Grumbach has very courteously sent microscopic preparations to us and we have verified the close resemblance which some of them bear to jagziekte and progressive pneumonia.

Coincident with these interalveolar infiltrations in both diseases, there is often a noticeable localized vascular engorgement.

The Alveolar Exudate.

This consists chiefly of large mononuclear cells which are like those already mentioned in the interalveolar tissue but are modified to some extent owing to enhanced phagocytic activity. Their appearance is the same in progressive pneumonia as in jagziekte in which they have been described in detail in a previous paper[4] which is supplemented by several photomicrographs. Attention may however be directed to their properties, as represented in the lower right hand corner of Fig. 8. There may be some polymorphonuclear leucocytes in addition, but the latter do not as a rule predominate in either jagziekte or progressive pneumonia except in later stages, when an acute pneumonic process is often met with (Figs. 11 and 12). Polynuclear giant cells, arising from the mononuclears, are often encountered. They are illustrated in Fig. 11 among the leucocytes. There is some desquamation of the epithelial cells. Coagulated blood protein is not abundant in the exudate.

The nature of the mononuclear cells, which are frequently known as "epithelioid cells" and "dust cells," is difficult to determine. Some investigators[10–13] believe them to be epithelial cells which have hypertrophied and desquamated into the alveolar lumina. Others incline to the view that they are of hematogenous origin. Permar[6] stimulated the production of these cells by injecting into the air spaces finely divided foreign material and followed all stages in their migration, having

[9] Grumbach, A., *Bull. Assn. franç. étude cancer*, 1926, xv, 213.
[10] Briscoe, J. C., *J. Path. and Bact.*, 1908, xii, 66.
[11] Sewell, W. T., *J. Path. and Bact.*, 1918–19, xxii, 40.
[12] M'Fadyean, Sir John, *J. Comp. Path. and Therap.*, 1920, xxxiii, pt. 1, 1.
[13] Mavrogordato, A., *J. Hyg.*, 1918, xvii, 439.

first marked them by vital staining with pyrrhol blue. He found no evidence that the nucleated or non-nucleated respiratory epithelial cells, described by Ogawa,[14] took up the dye. Lewis, Willis and Lewis[15] closely correlated the types of cells observed in experimental pulmonary tuberculosis as seen in sections and with the aid of supravital stains. They discovered that the epithelioid cells, both in the tubercles and within the lumen of the alveoli, resembled the large mononuclears (macrophages or monocytes) of the blood in many details of their structure (nuclei, centrosomes, mitochondria, neutral red granules) as well as in their phagocytic properties.

In jagziekte we believe that these large mononuclear cells may in the vast majority of cases be distinguished from desquamated respiratory epithelial cells by the following criteria:

1. Their nuclei are often kidney-shaped (and may thus be contrasted with the spherical nuclei of the epithelial cells) and by staining with iron-hematoxylin a centrosome, or a diplosome, is revealed on the side of the nuclear concavity.

2. The centrosome is always centrally placed (remote from the periphery of the cell) and the cytoplasmic granules about it are arranged radially. In hematoxylin and eosin preparations the position of the centrosome is marked by an area of cytoplasm which stains less intensely. Methods for the supravital staining of blood elements[16, 17] were not employed and it is doubtful whether they would be helpful in this case, because the question of the source of the cells thus examined (*i.e.* from the alveolar lumina, or ruptured alveolar capillaries, or interalveolar tissue) would remain.

3. There are many rod-like mitochondria in the cytoplasm accompanied by droplets of fat which may be colored with Sudan III. Both of these components are difficult to find in unaltered epithelial cells.

4. The mononuclear cells are often present in such enormous numbers as to suggest some almost inexhaustible reservoir of origin, such as the blood stream, as contrasted with the limited surface of the alveolar walls.

5. They are actively phagocytic for just those substances which macrophages are known to take up, and, on general principles, it would seem unlikely that desquamated epithelial cells would exhibit this property. In all other parts of the body desquamating epithelial cells are either dead or dying.

6. They appear within the alveoli before the epithelial cells hypertrophy and multiply *in situ*, or extend over the internal alveolar surface from restricted clusters, if we accept Ogawa's conclusion regarding their normal distribution.

7. What appear to be actual stages in the entry of these mononuclears into the

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[14] Ogawa, C., *Am. J. Anat.*, 1920, xxvii, 333.

[15] Lewis, M. R., Willis, H. S., and Lewis, W. H., *Bull. Johns Hopkins Hosp.*, 1925, xxxvi, 175.

[16] Cowdry, E. V., *Internat. Monatschr. Anat. u. Physiol.*, 1913–14, xxxi, 267.

[17] Simpson, M. E., *Univ. Calif. Pub. Anat.*, 1921, i, 1.

alveolar lumina, may in rare instances be seen and identified by their position and the hour-glass-like shape of their nuclei, when they are fixed half inside and half outside the wall of the alveolus (foot-note 4, Fig. 10).

8. In more advanced stages of the disease, when the epithelial proliferation has become extensive, so that all the alveoli are lined by cubical or columnar cells, the number of large mononuclears within the alveolar lumina is markedly diminished (see Figs. 5 and 6). This decrease in the mononuclears as far as can be determined is coincident, or follows immediately after, a constriction of the blood vessels caused by the pressure of the proliferating epithelial tissue and by interalveolar fibrosis. If the mononuclear cells were produced, for the most part, by the desquamation of epithelial cells, one would expect them to appear when the epithelial cells are most abundant instead of at a time when few if any of them may be seen and none of them has undergone any noticeable preparatory hypertrophy.

9. Some epithelial cells do, however, unquestionably desquamate. They break away from the alveolar wall in groups, seldom singly, by the operation of some factor or factors which inhibit or destroy their cohesive properties. Frequently the entire hypertrophied layer of alveolar epithelium splits away from the wall of the alveolus and comes to occupy a position within it as indicated in Fig. 7 of jagziekte and in Fig. 8 of progressive pneumonia.

The origin of most of the mononuclear cells seems, thus, to be the same—namely, from the contents of the alveolar capillaries and the interalveolar tissue—in both jagziekte and progressive pneumonia, although we have not examined the centrosomes, mitochondria and neutral fat in the latter condition.

The Epithelial Proliferations.

There is some question whether distinctive proliferations of the respiratory epithelium are to be observed in all cases. While Theiler[18] considers them to be definitely indicative of jagziekte, Mitchell[3] failed to find them in two out of fifteen cases and did not refer either to their presence or absence in three others. In progressive pneumonia Marsh has not always found them. In general they seem to be of slightly more constant occurrence in jagziekte.

In both conditions they commence in isolated foci like those represented in Figs. 2 and 3. That they originate through a metamorphosis of alveolar epithelium may be readily ascertained by the study of serial sections, which shows that they are often entirely separate and apart from the epithelium of the bronchioles. But the bronchiolar

[18] Theiler, Sir Arnold, personal communication.

epithelium also proliferates, though less extensively. These foci of overgrowth of the alveolar epithelium later merge with the result that large masses of tissue become almost (or in fact) adenomatous (Figs. 5 and 6) and present an appearance resembling superficially a mammary gland regenerating during lactation. The cells are sometimes, in the older growths, quite atypical in their properties (foot-note 5, Fig. 12). They are generally arranged in a single layer but in both diseases many of them may become superposed so that irregular masses of cells result. Within the bronchiolar proliferations dense and circumscribed clumps of polymorphonuclear leucocytes, each from about ten to twenty cells in number, may occasionally be distinguished.

Mitotic figures are of comparatively rare occurrence but appear to be a little more numerous in progressive pneumonia. Nuclear appearances indicative of widespread amitotic multiplication of the epithelial cells were seldom observed in either disease.

In general, the proliferations seem to be somewhat more luxuriant and tumor-like in jagziekte, but cases of progressive pneumonia are often observed in which they are equally highly developed and constitute just as conspicuous features of the disease. They are slightly invasive in both (foot-note 5, Fig. 11), but never metastasize, even to lymph glands within the thorax. Other parts of the body are not affected except by the general cachexia.

The proliferations are obviously secondary to the infiltrative and exudative changes already referred to. In common with hyperplasias of respiratory epithelium in man[19,20] they constitute a sort of aftermath of a preexisting infection. But they are undoubtedly more marked than any which have been reported in man, except in the case of definite neoplasms in which, however, the distinctive and primary interalveolar changes, already referred to, are generally absent.

Other Lesions.

A comparison of Fig. 7 of jagziekte and Fig. 8 of progressive pneumonia with Figs. 5 and 6 of earlier lesions will illustrate the commenc-

[19] Winternitz, M. C., Mason, I. M., and McNamara, F. P., The pathology of influenza, New Haven, 1920.

[20] Hart, C., *Deutsch. Arch. klin. Med.*, 1904, lxxix, 108 (measles).

ing interalveolar fibrosis which becomes very marked in advanced stages of both diseases and leads to widespread consolidation. The occurrence of small definitely outlined areas of myxomatous tissue is also typical (Figs. 9 and 10).

The pneumonic process is, as far as we can ascertain, of fundamentally similar nature in jagziekte and progressive pneumonia. Corresponding parts of the lungs are affected and it spreads in the same way, bringing about eventually the death of the animal.

Comparison of Jagziekte and Progressive Pneumonia with Verminous Pneumonia.

Through the kindness of Sir John M'Fadyean we have had the privilege of examining also a specimen from a case of verminous pneumonia in sheep. On the basis of this specimen and of his published description[12] it would apparently be difficult, through microscopic examination alone, to distinguish the lesions of this disease from those of jagziekte, unless perhaps very abundant material were available. Like jagziekte, the lesions of verminous pneumonia differ from those of progressive pneumonia chiefly by somewhat lesser involvement of the interalveolar tissue and by slightly more intense epithelial proliferation. The presence of nematodes and of their ova within the lungs in verminous pneumonia and their undoubted rôle in the production of the disease, as discovered by M'Fadyean, led us to make a very careful search for animal parasites in both jagziekte and progressive pneumonia, which was, however, unavailing.

DISCUSSION.

The pathogenesis of the lesions which we have briefly described in jagziekte and progressive pneumonia suggests the conclusion that the lungs have been subjected over a rather long period of time (4 to 8 months) to a variety of injurious influences, mechanical, in the form of foreign material, and infective, through bacterial invasion. It seems indeed surprising that similar conditions have not been reported from still other localities. With the possibility in mind that, although such accounts have not been published, the diseases may nevertheless occur, letters of inquiry were sent to Professor José Lignieres at Buenos Aires (Argentina) and to Professor Harold A. Woodruff at Melbourne (Australia). The answers received, together with a letter from Dr. H. R. Seddon of the Department of Agriculture, New South Wales, indicate that no cases of disease resembling jagziekte

or progressive pneumonia have thus far attracted attention in these great sheep-raising countries.

Definite predisposing influences are unknown, but that a dietary factor may nevertheless be involved is indicated by Theiler's study of jagziekte in horses,[21] which he found to be caused by the eating of a poisonous plant (*Crotalaria dura*), but as yet there are no observations available which point in this direction in the case of these two destructive diseases of sheep. Nor do we know just how closely jagziekte of horses resembles jagziekte of sheep. From Theiler's description it would appear that the epithelial proliferations are alike; but it is no longer possible to collect material for comparison without experimentally producing the disease by feeding; because as soon as its cause was discovered, preventive measures were taken which have completely banished it from the Union of South Africa.

In order to secure advice as to whether the action of some foreign protein might, in fact, constitute a specific predisposing factor in jagziekte, specimens were sent to Dr. C. F. Hoover of Western Reserve University. Dr. Hoover states that it is conceivable that repeated inhalations of protein may be responsible for such a chronic condition, but that if this were the case one would expect the attacks of respiratory distress to be paroxysmal, which is not characteristically so in either jagziekte or progressive pneumonia.

In regard to the precise cause of the epithelial proliferations, very little may be said. Robertson's[22] theory that they are produced by a malaria-like microorganism has received no support. It seems likely, when we take into consideration the occurrence of similar proliferations in verminous pneumonia,[12,23] and the multiple adenomata reported by Ebner,[24] that the respiratory epithelium of sheep is unusually prone to undergo this change. That epithelial proliferations are commonly produced by bacterial infections[25] and by a variety of experimental agencies[26] is well known. Yet as far as our

[21] Theiler, Sir Arnold, *7th and 8th Rep. Director Vet. Education and Research*, Union of South Africa, 1918, 59.

[22] Robertson, W., *J. Comp. Path. and Therap.*, 1904, xvii, 214.

[23] Hofman, V., *Časop. lék. česk.*, 1923, lxii, 65; abstracted in *J. Am. Med. Assn.*, 1923, lxxx, 1279.

[24] Ebner, A., *Z. Tiermed.*, 1899, iii, 161.

[25] Teutschlaender, *Centr. allg. Path. u. path. Anat.*, 1914, xxv, 424.

[26] Fischer, B., *Frankf. Z. Path.*, 1922, xxvii, 98.

present information concerning jagziekte is concerned it is still possible that the proliferations may arise through the action of a single ultravisible virus, as claimed by Theiler;[18] but we do not believe that the entire disease complex is caused by such a virus. It is interesting, in this connection, to recall that Roux[27] discovered the existence of an adenomatous proliferation of the respiratory epithelium in sheep-pox, but unfortunately he only referred to it very briefly and gave no illustrations.

If the proliferations in jagziekte and progressive pneumonia are in truth neoplastic, it is difficult to explain Mitchell's failure to transmit the disease by the inoculation of tissue fragments despite the fact that he did not avail himself of recent methods of intratesticular inoculation with a large series of animals. Perhaps the failure is due to the difficulty of producing experimentally the primary lesions, the nature of which we have stressed and which may be prerequisite to the growth of the tumor. Acceptance of the tumor hypothesis would render intelligible the fatal outcome in all recognizable cases of the diseases. Obviously, if we are dealing with a tumor, it does not mean that we are faced by one which of itself and independently spreads from diseased to healthy sheep; on the contrary, the view that we have to do with a neoplasm which comes in the wake of an acute infection or series of infections, most probably bacterial in nature, is not improbable.

SUMMARY.

As long as the exact etiology of jagziekte and of progressive pneumonia remains unknown, it cannot be said that they are identical, although on the basis of the observations detailed above, they certainly appear to be. It is extremely doubtful whether it is possible to distinguish between them. In the localities in which they occur, each is recognized as an infection quite distinct and apart from other better known pneumonias. Predisposing factors which lead to bacterial invasion are presumably of great importance in both. We have no precise information regarding the organisms concerned, but in both diseases, the initial changes are alike and occur in the interalveolar tissue and the subsequent proliferations of epithelium and the pneu-

[27] Roux, E., *Bull. Inst. Pasteur*, 1903, i, 49.

monia are of the same character and apparently follow in exactly the same sequence. Finally, in both diseases, there is much fibrosis and the animals inevitably die through depletion of respiratory area and pneumonia.

The view hitherto held, that jagziekte is a disease definitely restricted to the Union of South Africa, is thus rendered improbable. Nor does it appear likely that jagziekte is due to a single specific virus acting primarily upon the epithelial cells of the lungs, which is is likewise a conception widely accepted in South Africa.

EXPLANATION OF PLATES.

To facilitate comparison of the two diseases photomicrographs taken at a magnification of 240 diameters of approximately similar lesions are given in pairs, jagziekte on the left and progressive pneumonia on the right hand side.

PLATE 16.

FIG. 1. Interalveolar infiltration in jagziekte.

FIG. 2. The same in progressive pneumonia.

FIG. 3. Beginning epithelial proliferation in jagziekte.

FIG. 4. The same in progressive pneumonia with rather more interalveolar infiltration.

PLATE 17.

FIG. 5. More advanced proliferation in jagziekte.

FIG. 6. The same in progressive pneumonia.

FIG. 7. Desquamation of proliferated epithelium in jagziekte.

FIG. 8. Same in progressive pneumonia with less interalveolar change. The alveoli in the right lower corner contain typical large mononuclear cells.

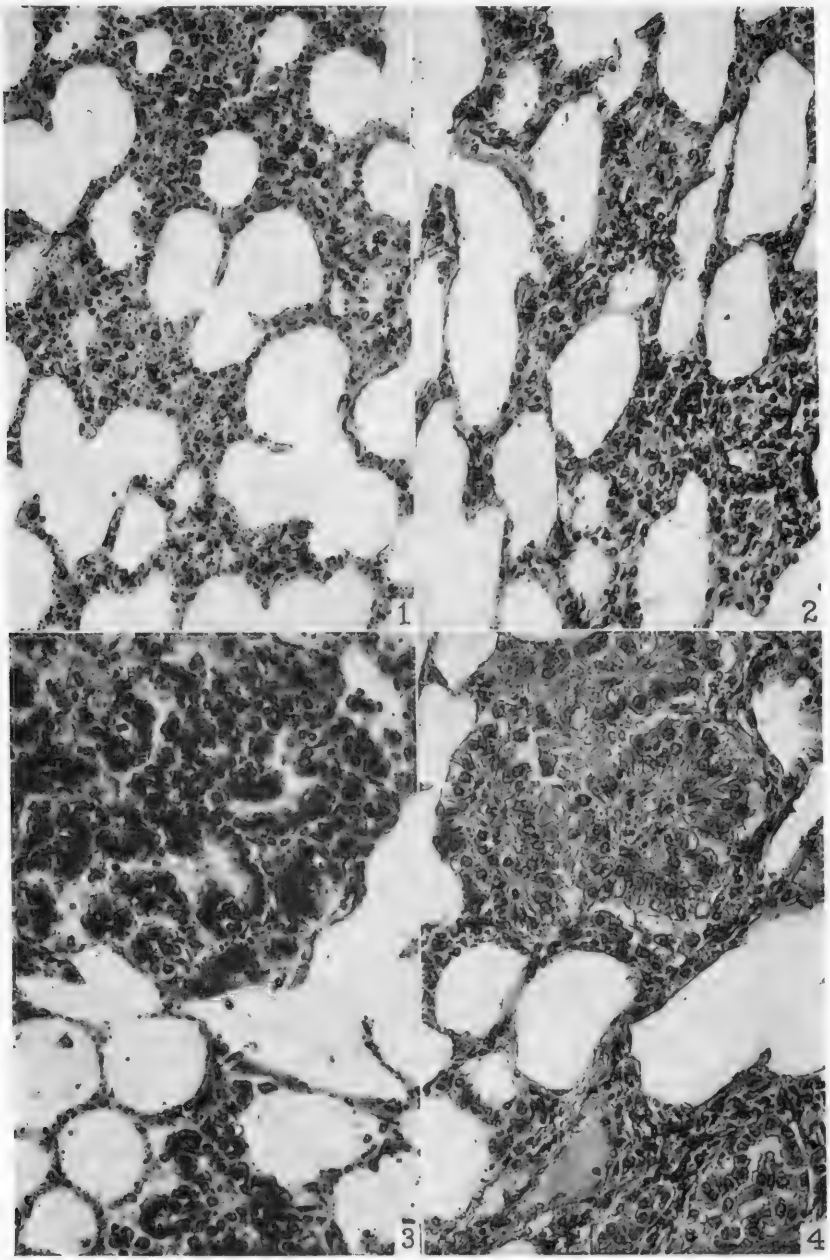
PLATE 18.

FIG. 9. Nodule of myxomatous tissue in jagziekte.

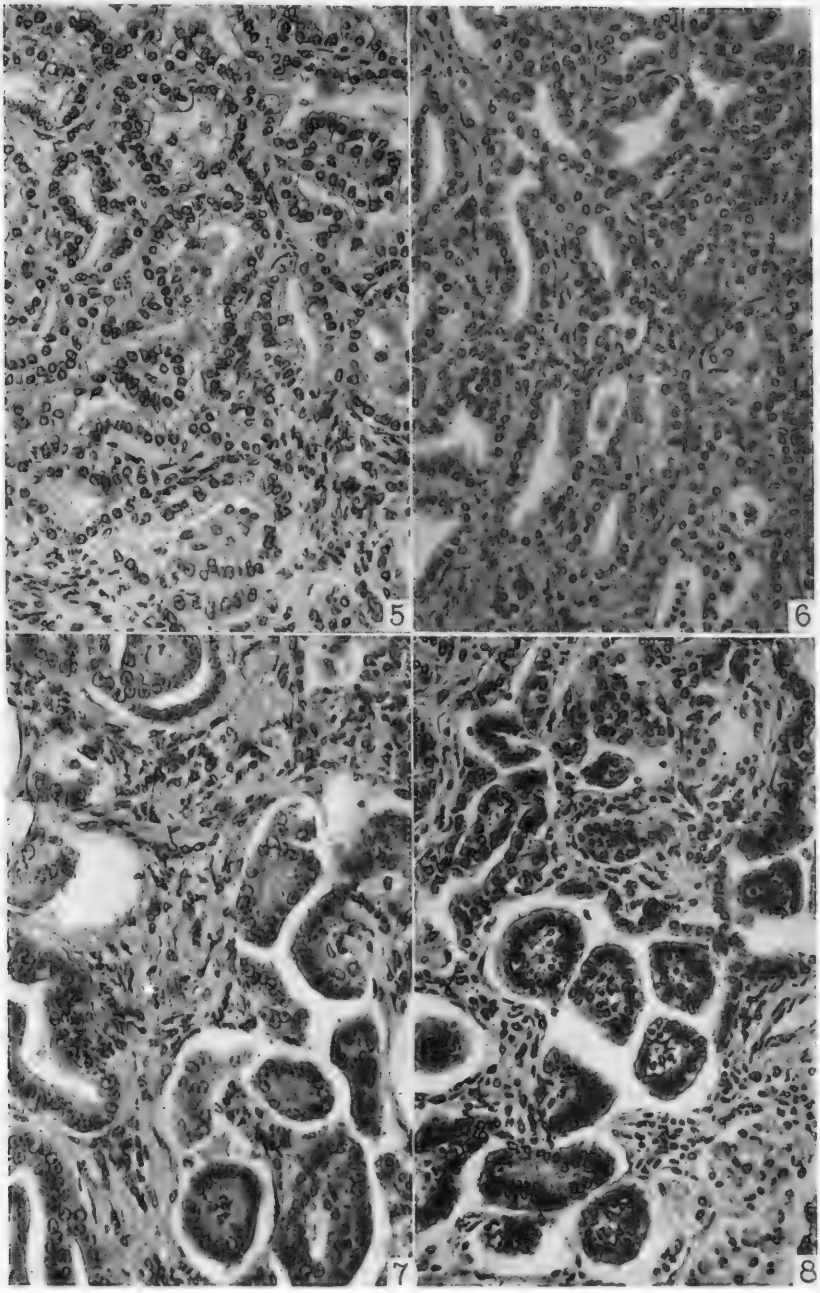
FIG. 10. The same in progressive pneumonia. It will be noted that the cells are larger because they have not been shrunken so greatly by the fixative.

FIG. 11. Masses of polymorphonuclear leucocytes in the lumina of bronchioles in jagziekte. Many polynuclear giant cells are visible.

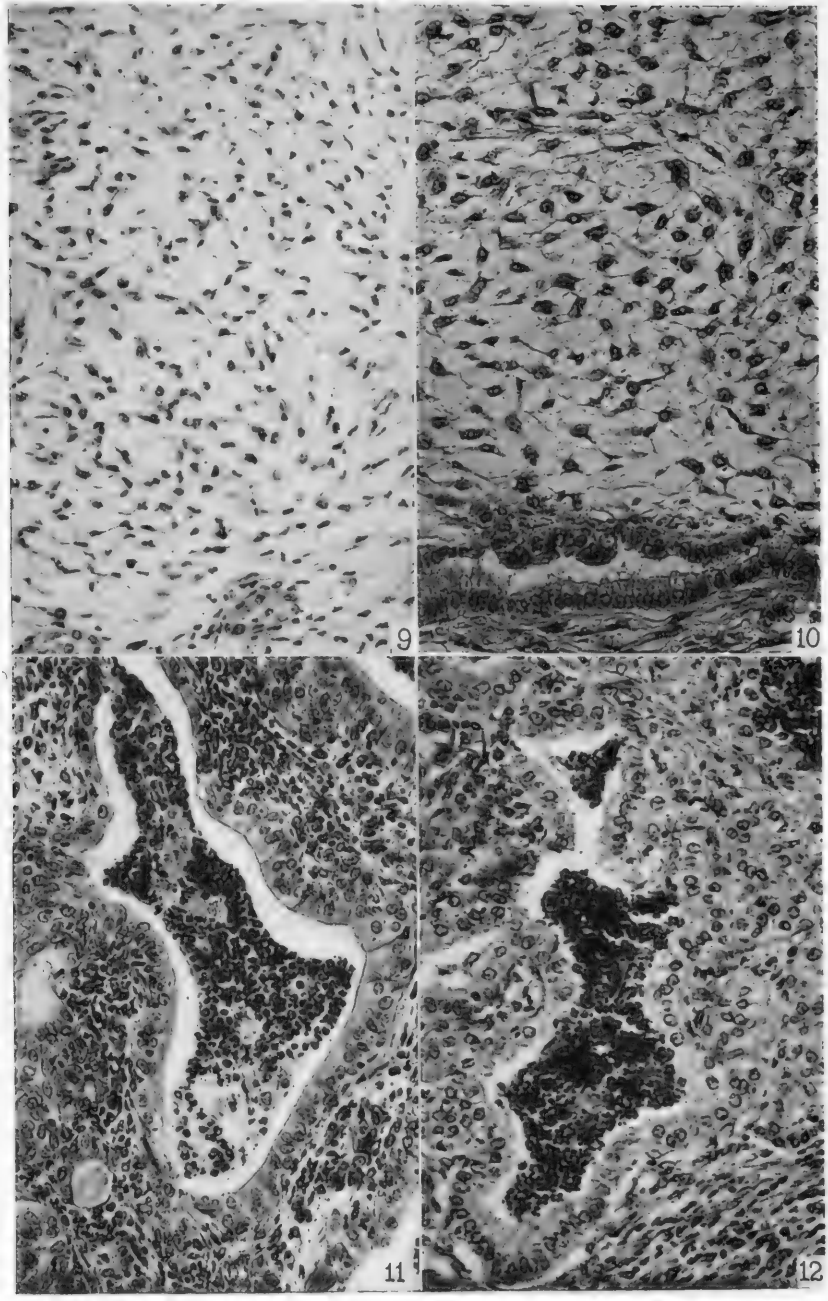
FIG. 12. The same in progressive pneumonia except that in this case the proliferated epithelium is rather lower, being more typical of the altered alveoli, and giant cells may be only indistinctly seen.



(Cowdry and Marsh: Jagziekte and pneumonia of sheep.)



(Cowdry and Marsh: Jagziekte and pneumonia of sheep.)



(Cowdry and Marsh: Jagziekte and pneumonia of sheep.)

ON THE TRANSPLANTATION OF THE GUINEA PIG SUPRARENAL AND THE FUNCTIONING OF THE GRAFTS.

By HENRY L. JAFFE, M.D.

With the Assistance of Alma Adler.

(*From the Laboratory Division, Hospital for Joint Diseases, New York.*)

Plate 19.

(Received for publication, November 30, 1926.)

In 1906 Elliott and Tuckett[1] reported their inability to transplant the guinea pig suprarenal homoplastically into the subcutaneous tissues of the abdominal wall. Within a few hours after grafting a half gland beneath the abdominal skin of a guinea pig the surrounding tissues swelled and appeared irritated. Next day the swelling increased, the skin over the transplant turned green, and a foul smelling, clear fluid transuded from between the skin sutures. The escape of the fluid relieved the irritation, the skin over the swollen area sloughed away, and the animal recovered. If the fluid did not escape, the irritation extended rapidly over the surface of the body, the animal's temperature fell, and it became comatose and died in 30 hours. The only organ showing noteworthy pathology was the suprarenal, the cortex of which was congested, hemorrhagic, and without the brown granules, while the medulla showed the absence of chrome staining.

To our knowledge the successful transplantation of the guinea pig suprarenal has not been reported, due, we felt, to the failure to remove the medullary tissue from the grafts. Taking this precaution we sought to obtain positive and functioning autoplastic transplants in this animal. All previous experience has shown that medullary tissue never regenerates, so that its removal is of no consequence in a study of suprarenal transplants.

[1] Elliott, T. R., and Tuckett, I., Cortex and medulla in the suprarenal glands, *J. Physiol.*, 1906, xxxiv, 361.

Methods.

Most of the guinea pigs used were born in the laboratory, and were between 2 and 3 months old at the beginning of the experiment, except for a few aged 5 or 6 months. Under ether anesthesia first the right suprarenal was completely removed through the dorsal route, and was kept in normal saline at 38°C. until the wound was closed. It was placed on a cork and cut longitudinally with a sharp razor blade into 4 or 8 segments. The medulla together with some of the adjacent cortex was removed from each segment with fine curved dissecting scissors, to insure extirpation of the epinephrine-containing tissues. Each segment of cortex was divided transversely, and the small pieces were washed for 15 minutes in the physiological saline. Meanwhile many pockets were prepared in the abdominal wall by puncturing the muscle with a cataract knife, and enlarging the openings by spreading an ordinary forceps in them. One fragment of suprarenal cortex was introduced into each pocket, the mouth of which was closed with a black silk marking suture. The skin was sewed with silk and covered with celloidin. 3 to 7 weeks later the left suprarenal was removed. All tissues were fixed in Zenker-formol, embedded in paraffin, serially sectioned, and stained with hematoxylin and eosin.

Data.

In 22 guinea pigs the right suprarenal was removed and autotransplanted into the abdominal muscle. 3 to 7 weeks later the left suprarenal was removed from 17 of these. Some animals died from operative shock, others from suprarenal insufficiency, and still others were killed in good condition to terminate the experiment. There were no toxic deaths attributable to the transplants, although irritation and swelling of the abdominal muscle frequently occurred. 84 per cent of our animals had 1 or more takes varying from nests of a few cells to masses several mm. in diameter when examined after 3 weeks. Although 8 to 16 transplants were inserted in each animal, at autopsy usually only 3 or 4 were found. In the guinea pig the percentage of transplants that take compared with the number of transplants inserted is quite small because the severity of the reaction following the transplantation influences the course of the regeneration.

In spite of the gaps in our series, the successive histological changes in guinea pig suprarenal transplants can be followed. Stages of degeneration and regeneration, followed either by the absorption of the regenerated transplant or its marked growth, have been observed.

After 3 days transplants show marked necrosis, only a few supra-

renal cells remaining at the periphery. These surviving cells show cloudy protoplasm with indistinct cell walls. The necrotic area is edematous and is infiltrated by some polymorphonuclear leucocytes and round cells. At the periphery of the transplant new blood vessels have appeared. About the transplant the tissue has become edematous, some polymorphonuclear leucocytes and round cells have infiltrated, and sprouting fibroblasts appear.

By 11 days the transplant area is very celullar with little debris remaining. Great numbers of polyhedral cells are present having large vesicular nuclei, prominent nucleoli, and hazy, granular, pinkish staining cytoplasm. These cells sometimes occur in small groups appearing like multinuclear giant cells. Connective tissue cells are seen between the regenerating suprarenal cells, and new blood vessels penetrate the transplant. Some of the regenerating suprarenal cells are dividing mitotically. The muscle about the transplant shows an intense interstitial inflammation, and muscle giant cells and pigmented cells are seen.

By the 22nd day the transplants diminish in size and appear macroscopically as tiny yellow-white specks which fuse in color with the fascia of the abdominal muscle. Microscopically groups of newly regenerated suprarenal cortical cells in glomerular formation are seen. In addition considerable connective tissue has formed between these cells. Large numbers of lymphocytes appear in some transplants at this stage, probably in those destined for rapid absorption.

During the 4th and 5th weeks regeneration is active, and the suprarenal cells increase by hypertrophy and hyperplasia, and glomerular and fascicular formations appear. The transplant areas still show considerable interstitial connective tissue. At about this time transplants show tendencies either to absorption or rapid growth. Absorption progresses slowly, taking many weeks, and is characterized by shrinking of the suprarenal cells, progressive lymphocytic infiltration and interstitial fibrosis, and the appearance of many pigmented phagocytes. During the course of this slow absorption many of the animals die of suprarenal insufficiency, evidently due to the inability of the transplants to maintain life.

On the other hand the transplants which show active growth enlarge by the multiplication of suprarenal cells mostly through amitotic

division while the interstitial connective tissue diminishes. Dark, actively growing suprarenal cells are mingled with lighter larger cells, and as regeneration proceeds the cells become larger and more fatty. After 100 days some transplants show the glomerular, fascicular, and reticular layers while the connective tissue and lymphocytes have disappeared. With further growth the transplants sometimes appear as masses of very large closely packed polyhedral cells with markedly vacuolized protoplasm. Some of these cells are completely replaced by fat. Between 100 and 200 days transplants may reach the size of 3 or 4 mm. in diameter. The larger transplants may show adenomatous nodules at the periphery in which the typical cortical arrangement of the cells is seen. The oldest transplant we studied was 276 days, and it showed no signs of degeneration or exhaustion atrophy.

We studied homotransplants in 5 animals and found that during the first 2 weeks the changes are the same as in autoplastic transplants. During the 3rd week an occasional clump of regenerated suprarenal cells is seen, but foreign body reactions with giant cells and lymphocytes are already in evidence. By the 7th week most of the homotransplants are completely absorbed.

Functioning of the Transplants.

From these experiments we obtained evidence that autoplastic transplants are capable of maintaining the life of guinea pigs in the absence of both main glands and suprarenal accessories. If the transplants are small or are undergoing absorption, they maintain the animal in a poor state, which may continue for weeks until the guinea pig dies of suprarenal insufficiency. In those instances where the transplants reach full development, guinea pigs are maintained normally for a sufficiently long time to warrant the assumption that they can live their normal life period. We have observed 1 transplanted guinea pig surviving suprarenalectomy in good condition for 9 months.

Of 17 doubly suprarenalectomized and transplanted animals 5 died from suprarenal insufficiency within 5 days after removal of the second gland. 1 death occurred on the 1st day, 1 on the 2nd, 2 on the 4th, and 1 on the 5th. The autopsies showed complete removal of both

main glands, and no macroscopic accessories. Transplants had been inserted from 24 to 59 days previously, and had been absorbed in all but 1 animal which showed some clumps of suprarenal cells in each of several transplants. They were apparently insufficient to maintain life. 1 guinea pig died from a transverse myelitis due to osteomyelitis of the thoracic spine on the 7th day after removal of the second gland; another died of a general infection following an abortion on the 15th day; a 3rd died from pneumonia on the 24th day. These 3 had been transplanted 29 to 59 days previously, and each had active regenerating transplants.

One guinea pig was sacrificed in good condition 13 days after removal of the left gland and 57 days after insertion of the transplants. The autopsy showed complete bilateral ablation of the suprarenals, no accessories, and numerous pin-head-sized positive transplant areas which when sectioned showed groups of vascularized cortical cells.

Following the removal of the second gland, 4 guinea pigs died of chronic suprarenal insufficiency, 1 each on the 41st, 46th, 52nd, and 57th day. The transplants had been inserted 85 to 104 days previously, and on histological examination were degenerated and fibrotic. No accessories were found in these animals.

One animal killed 220 days after the removal of the second gland showed a large cortical accessory. No transplants were present. The remaining 3 animals were killed, 2 on the 124th, and 1 on the 221st day after the removal of the second gland. Each had several large vascular transplants which were apparently physiologically active, maintaining the animals in good condition. 1 of these animals had a 3 mm. accessory.

In summary we can present 11 guinea pigs upon which we base our conclusions regarding the functioning of the transplants. These animals after suprarenalectomy and transplantation either died of suprarenal insufficiency or were killed to terminate the experiment months after transplantation. At autopsy no accessory cortical tissue was found in any of these animals. Of the 11 animals, 6 lived longer than 40 days after the removal of the second gland. These figures stand in marked contrast to the average survival time given in the literature for double suprarenalectomized guinea pigs, and which we ourselves have found, that is, 3 or 4 days. They indicate that

transplants are capable of prolonging the life of suprarenalectomized guinea pigs or of maintaining them indefinitely.

DISCUSSION.

Irritation phenomena occurred in our experiments even when suprarenal cortex alone was transplanted, but were different in their appearance, severity, and consequence from those described by Elliott and Tuckett who transplanted both cortex and medulla. The cortical fragments were definitely palpable as firm plaques for about 3 days after their insertion due to the absorption of water by the lipoid-rich tissue. By the 4th or 5th day serosanguineous fluid had collected underneath the abdominal wound stitches of nearly all the animals. Sometimes 10 cc. of a bloody fluid could be expressed. Frequently on the 6th or 7th day the wound opened spontaneously, the skin edges sloughed out, and the transplants were destroyed. The edema and inflammatory reactions around the remaining transplants were followed by connective tissue growth, which impaired the vitality of the transplant during its organization through pressure and interference with the circulation. The result is frequently absorption of the transplant, but even if the transplant is not absorbed its growth is usually interfered with.

The reactions that occur with suprarenal transplantation in the guinea pig have not been conclusively explained. Elliott and Tuckett believed that the edema and solution of the guinea pig tissues were caused by some substance in the medulla, not epinephrine, and not present in all mammalian suprarenals. They reported that the irritant substance was diffusible and destroyed by heating to 65°C.

They excluded epinephrine as the toxic agent because the subcutaneous injection of 1 mg. in 1 cc. of salt solution caused a skin slough, but never the inflammatory reaction with edema and corrosion of the tissues such as is produced by the medulla. Even in lethal doses epinephrine did not cause an inflammatory reaction. It was further shown that this reaction did not follow the grafting of the whole gland on the peritoneum, or between the muscle and peritoneum. Neither do irritation phenomena follow the transplantation of the guinea pig suprarenal beneath the skin of the rabbit, rat, or cat. It

occurs only when the whole suprarenal is transplanted, auto-, homo-. or heteroplastically beneath the skin of a guinea pig. The appearance of these changes is peculiar to the suprarenal, for transplantation of the kidneys, spleen, thyroid, thymus, bone, and cartilage into the subcutaneous tissues of the guinea pig is not followed by edema or corrosion. Nor does transplantation either auto- or homoplastic of the suprarenal of the dog, cat, rabbit, or rat produce this phenomenon.

We believe that the milder reactions we observed following the transplantation of suprarenal cortex and the acute reactions described by Elliott and Tuckett are identical and are induced by the same irritating substance which however is present in greater concentration in the medulla. We believe that this substance has entered the cortex from the medulla through manipulation of the gland during its removal. We cannot agree with Elliott and Tuckett that the substance is neither epinephrine nor related to it, for the injection of epinephrine causes sloughing of the tissues. The difference between the reaction which follows the introduction of epinephrine alone, and that following the grafting of the entire suprarenal may be one of degree, due to the poulticing effect exercised by the necrotic transplant. Marine and Sandberg in some unpublished work concluded that epinephrine was the substance responsible for the phenomenon reported by Elliott and Tuckett. Some peculiarity of the guinea pig's subcutaneous tissues and abdominal muscles may in part be accountable for this idiosyncracy.

In our experiments the right gland was first removed and transplanted, and some weeks later the left gland was removed. This is the satisfactory way to study transplantation in an animal so susceptible to double suprarenalectomy. We cannot say whether a greater number of transplants would have taken and grown had a suprarenal insufficiency been induced by the removal of more than one gland at the initial operation, but it seems that the take of the suprarenal transplant is not necessarily dependent upon an insufficiency. 3 weeks elapse before the transplants regenerate, and at about this time the second gland is removed in most cases. This must be a stimulus for the growth of the transplants. Some respond and grow, while others degenerate even when so stimulated.

CONCLUSIONS.

1. The suprarenal cortex of the guinea pig can be transplanted autoplastically into the abdominal wall and may remain for months, growing to fairly large size.

2. The suprarenal cortex of the guinea pig can be transplanted homoplastically but these transplants usually degenerate after a few months.

3. Small autoplastic transplants are capable of maintaining the life of a completely suprarenalectomized guinea pig for weeks and large transplants maintain the animal indefinitely in good condition.

4. Irritation phenomena follow the grafting of suprarenal cortex due to the entrance of epinephrine into this tissue during the manipulation involved in removal of the gland.

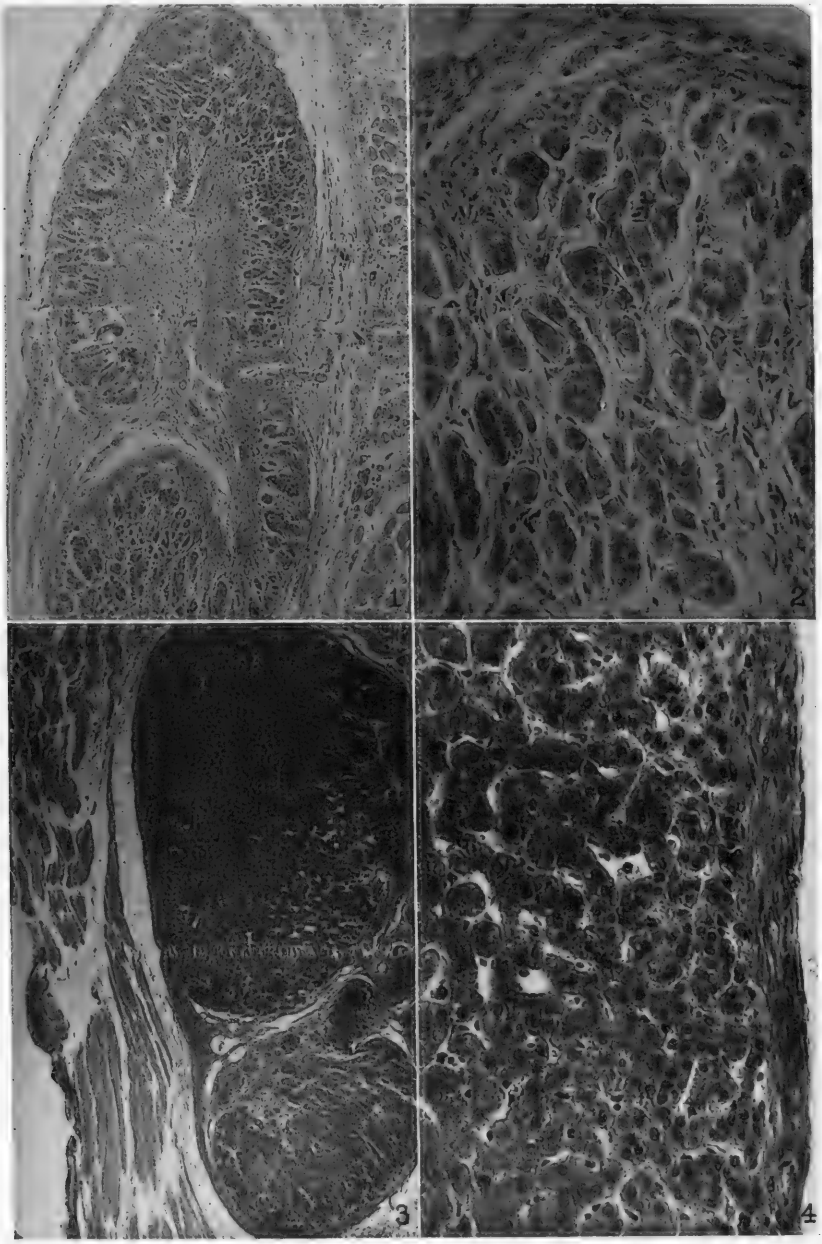
EXPLANATION OF PLATE 19.

FIG. 1. Low power of a 59 day autotransplant. Regeneration is progressing actively though there is considerable scarring in the center of the graft.

FIG. 2. High power of Fig. 1 showing cortical cells in glomerular formation.

FIG. 3. Large completely regenerated suprarenal transplant 142 days after insertion.

FIG. 4. High power of Fig. 3 showing closely packed, highly vacuolated cortical cells.



(Jaffe: Transplantation of guinea pig suprarenal.)

THE INFLUENCE OF THYROIDECTOMY, SPLENECTOMY, GONADECTOMY, AND SUPRARENALECTOMY UPON THE DEVELOPMENT OF EXPERIMENTAL ATHEROSCLEROSIS IN RABBITS.*

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(Received for publication, November 30, 1926.)

Ignatowski (1) showed in 1908 that in rabbits the prolonged feeding of foods of animal origin caused atherosclerosis of the aorta. He believed that the proteins were responsible for the intimal changes. It was subsequently found by Stuckey (2) and by Wesselkin (3) that only cholesterol-containing substances produced these arterial changes and it was finally determined that cholesterol was the essential constituent when Anitschkow and Chalatow (4) and Wacker and Hueck (5) produced atheromata of the aorta by feeding pure cholesterol in oil to rabbits. Later Aschoff and his coworkers (6) demonstrated that the anisotropic crystals deposited within the areas of intimal swelling and softening were cholesterol esters.

The similarity of experimental atheromata to those seen in humans was pointed out by various authors, especially Anitschkow (7), Aschoff (8), Zinserling (9), Klotz (10), and Bailey (11). It was recognized, as has been emphasized by Bailey (11), that although the experimental atheromata are practically identical with those seen in man, constantly associated with the artificially produced lesions are fatty deposits in other tissues such as spleen, liver, and kidneys not usually accompanying atherosclerosis in man. Anitschkow (12) therefore fed to rabbits over long periods of time (about 2 years) diets containing small doses of cholesterol, producing only very slight or no hypercholesterolemia. At autopsy these animals showed atheromata but no deposits in the other organs.

* Presented in abstract before the American Society for Experimental Pathology, Cleveland, December 28, 1925.

Much additional work on experimental atherosclerosis has been done and numerous reports published from various sources, especially Russia, Germany, and this country. Historical reviews have been included in several of the older and more recent papers. Therefore, no attempt will be made at this time to cover this phase of the subject and those interested are referred to the publications of Rickett (13), Dewey (14), Schönheimer (15), Bailey (11), and Chuma (16).

Comprehensive descriptions of the intimal lesions as they occur in man and in rabbits following cholesterol feedings have been published by various authors, especially those already mentioned, and are so well known that we shall merely refer to them briefly. The lesions observed by us were identical with those reported by these workers and may be described as follows:

Blocks were taken from the thoracic aorta, usually at the arch, and fixed in 10 per cent formalin. Celloidin sections were stained with hematoxylin and eosin, frozen sections with Sudan III and hematoxylin, and unstained sections, mounted in glycerol jelly, were examined with the polarizing microscope. In some instances paraffin sections were stained with Weigert's elastic tissue stain and counterstained with Van Gieson's stain.

Because this was a comparative study we classified the lesions into (1) early atheromatosis, (2) moderate, and (3) severe atheromatosis.

1. Early Atheromatosis.—These were the earliest changes observed. There were sparsely distributed swellings of the vessel wall which macroscopically appeared as punctate elevations on the intima. Microscopically they consisted of local thickenings of the intima in which were present large irregularly shaped cells occurring in one or several layers and containing a substance which stained with Sudan III, a small amount of which was anisotropic. The internal elastic membrane and the outer layers of the aortic wall showed no alterations at this stage.

2. Moderate Atheromatosis.—Macroscopically the lesions were more numerous and larger. Microscopically at this stage there was further thickening of the intima with an increase in the number of large irregular "foamy" cells. The internal elastic membrane was more prominent and showed some separation of its fibers. Within the media there was a small amount of fat-staining material which was anisotropic.

3. Severe Atheromatosis.—In severe atheromatosis the lesions extended over the greater part of the intimal surface of the aorta. Microscopically the intima was much more thickened and the large foamy cells were still more numerous. Fibroblasts were visible. The Sudan-staining anisotropic material was more abundant both in the intima and media. There was much more separation of the elastic fibers at this stage.

More recent work by Murata and Kataoka (17) and by Chuma (16) has shown that castration tends to facilitate the development of atheromata after lanolin (cholesterol) feeding. Murata (18) reported further that thyroid administration to these rabbits inhibits atheroma formation.

Because of their susceptibility to the development of atherosclerosis rabbits have been used in these experiments. It has been possible also to produce the same changes in guinea pigs (Anitschkow and Chalatow (4), Schönheimer (15)) and in omnivorous animals, especially after castration (Löwenthal (19)).

The possible occurrence of spontaneous atheromata in rabbits is of the greatest importance in this work. There are many factors which may be responsible for the development of the condition, notably, age of the animal, hygienic conditions, previous infections, and diet. The frequency of spontaneous atherosclerosis has, therefore, been observed and commented upon by various authors. The findings, however, have been in wide disagreement. For example, Miles (20) found 17 out of 49 rabbits to have spontaneous atheromata while Steinbiss (21) could find none in 500 rabbits examined. Accordingly van Leersum (22) in 1914 tabulated the findings of various authors up to that time. Of 1937 rabbits examined spontaneous lesions were found in 49 instances. In addition to these Ophüls (23) has reported none present in 50 rabbits while Weinberg (24) found up to 9 per cent of 692 rabbits to have spontaneous atheromata.

Therefore, it is of the greatest significance that all the rabbits used in the experiments reported in this paper were of known age, were born and reared in this laboratory, lived under practically identical conditions since birth, and were apparently healthy and vigorous when selected for the experiments. Furthermore, it is noteworthy that of more than 250 rabbits which came to autopsy in this laboratory, a number of them litter mates of the rabbits used in these experiments, and which were examined for atheromata, only 1 had spontaneous lesions in the aorta. This rabbit had long standing snuffles and showed extensive chronic pulmonary disease at autopsy.

Cholesterol has been administered in various forms for the production of hypercholesterolemia and atherosclerosis. It has been given in pure form by intravenous injection in sesame oil (Adler (25)),

emulsified with sodium oleate (Klotz (10)), intraperitoneally in aqueous emulsion (Dewey (14)), and by feeding cholesterol-containing substances such as egg yolk, brain substance, dried liver (Anitschkow (7), Stuckey (2), Steinbiss (21), Bailey (11), and others). Lanolin (adeps lanæ hyd.) first used by Kon (26), contains cholesterol in large quantities and has been found to be an excellent means for introducing this substance into the body by feeding.

In a previous series of experiments (unpublished) we used the aqueous emulsion described by Dewey (14) intravenously and intraperitoneally but with unsatisfactory results. In the experiments herein reported we fed the lanolin in oil in addition to the regular laboratory diet of alfalfa hay, oats, and vegetables. The dose of lanolin used by us was 4 gm. daily in 12 cc. cottonseed oil. The mixture was heated to about body temperature and fed by pipette.

As has already been pointed out it has been found that cholesterol plays an essential rôle in the formation of experimental atheromata of the aorta. We have, therefore, studied some of the factors which are believed to influence the metabolism of this fatty substance and upon which might depend its abnormal deposition within the intima of the aorta. It is the purpose of this paper to present evidence to show the influence of feeding lanolin in oil on the production of experimental atherosclerosis in (1) normal rabbits, (2) thyroidectomized rabbits, (3) gonadectomized rabbits, (4) splenectomized rabbits, and (5) suprarenalectomized rabbits.[1]

In this report are included only the 44 animals which lived over 25 days. We excluded from the series 10 rabbits which died of marasmus due to toxicity of cottonseed oil and 4 which died of acute suprarenal insufficiency.

1. Normal Rabbits.—We found that rabbits with all organs intact sacrificed after 110 days of feeding with lanolin in oil showed early atheromata in the aorta. 3 rabbits sacrificed after 90, 95, and 100 days of the feeding respectively showed no atheromata. The others, killed after 110 days or longer, all showed atheromata.

2. Gonadectomized Rabbits.—Confirming Kon, Murata, and Chuma, we observed that gonadectomized rabbits developed atheromata more

[1]All operations were performed under ether anesthesia.

readily than those with all organs intact. After 50 to 60 days of feeding with lanolin the castrates showed early atheromata. There were 2 exceptions: 1 sacrificed after 50 and the other after 90 days showed no atheromata.

TABLE I.

Number of rabbits in group	Operative procedure	Period of lanolin feeding	Degree of atheromatosis	Remarks
		days		
10	None	110–120	Early	
6	Gonadectomy	50–60	Early	Two fed 50 and 90 days showed no atheromata
7	Splenectomy	50–60	Early and moderate	
8	Thyroidectomy	40–50 70	Moderate Severe	
6	Double suprarenalectomy	85–100 110	None Early	Largest thyroids 8 to 10 times normal size. Lymphoid overgrowth
1	Thyroidectomy and double suprarenalectomy	50	None	
1	Thyroidectomy and splenectomy	50	Severe	
3	Double suprarenalectomy and splenectomy	45 106–110	None Early	
2	Gonadectomy and splenectomy	100	Severe	

3. Splenectomized Rabbits.—After 50 to 60 days of feeding with lanolin in oil the splenectomized rabbits showed early and moderate atherosclerosis. The lesions were more extensive than those seen in the castrated animals which were fed over equal periods of time.

4. Thyroidectomized Rabbits.—Thyroid-deficient rabbits showed

moderate atheromata after 40 to 50 days and severe atheromata after 70 days of feeding with lanolin. This group was the most susceptible.

5. Suprarenalectomized Rabbits.—10 doubly suprarenalectomized rabbits were used. Of these 1 was also thyroidectomized and 3 splenectomized. Of the first 6, 5 were fed between 85 and 100 days. None of these showed atheromata. The remaining one was sacrificed after 110 days of feeding. Early atheromata were present. The thyroidectomized and suprarenalectomized rabbit was fed 50 days. The aorta was free from atheromata at autopsy. Of the 3 splenectomized and suprarenalectomized, 1 fed 45 days on lanolin showed early atheromata.

At the same time, for controls, 1 thyroidectomized and splenectomized rabbit was fed 50 days and 2 gonadectomized and splenectomized rabbits were fed 100 days. At autopsy all 3 showed severe atheromata of the aorta.

DISCUSSION.

We find, as have others, that deposition of cholesterol or cholesterol esters within the intima of the aorta initiates the cellular alterations which result in the formation of atheromata. We have readily produced it experimentally in rabbits by inducing prolonged alimentary hypercholesterolemia. We find further that thyroidectomy, splenectomy, and gonadectomy each facilitate and accelerate the development of this condition within the aorta. The thyroid-deficient rabbits are most susceptible and the splenectomized group are slightly more susceptible than the castrates.

The question arises: Why does removal of the gonads, the spleen, or the thyroid facilitate the development of experimental atheromata? In the case of the spleen we may be eliminating a possible storehouse for excess cholesterol because it sometimes undergoes very marked enlargement in the presence of prolonged lipemia. This is well illustrated in experimental lipemia and clinically in Gaucher's disease and in some cases of diabetic lipemia (1, 27, 5, 15, 28, 29, 30, and others). The relation of the spleen to the reticulo-endothelial system and its function in both the destruction of old red blood cells and the formation of new erythrocytes emphasizes the possibility that the spleen not only stores cholesterol but also utilizes it. Thus also it has been

found that splenectomy alone is followed by hypercholesterolemia (31–39).

Concerning the relation of the gonads to cholesterol metabolism very little is known. Their removal, however, may give rise to a slight hypercholesterolemia (40, 41). Certainly they do not store the fatty substance in any appreciable amount although the interstitial cells may appear more prominent in prolonged hypercholesterolemia. To what extent cholesterol is utilized in ovarian or testicular function still remains to be demonstrated. Clinically, menopause cases are said to show a tendency to develop hypercholesterolemia (42). One other possibility deserves emphasis, however, namely, the interrelation of the sex glands with the thyroid. It appears that a synergistic functional relationship operates between the gonads and the thyroid. For example, gonadectomy may cause a slight drop in heat production and even involution of the thyroid (43–49). Also, it is known that at those periods when the sex glands are the seat of both physiological and morphological alterations (puberty, pregnancy, climacteric) the thyroid may likewise undergo striking changes, sometimes to pathological extents so that exophthalmic goiter, simple goiter, or myxedema (Gull's disease) may result. In all probability, therefore, the importance of the gonads in cholesterol metabolism is dependent upon the interrelationship which exists between the sex glands and the thyroid (46, 50).

The thyroid on the other hand plays what appears to be an essential rôle in the metabolism of the fats and fat-like substances, including cholesterol. Exactly what processes in the burning of fats are dependent upon the presence of thyroxin for their completion cannot be stated at the present time. There is good evidence, however, that high fat diets cause increased thyroid activity. Indeed, unless iodine is administered to prevent it, thyroid overgrowth soon results. This has been amply illustrated in the work of Marine and Lenhart (51), McCarrison (52), the Mellanbys (53), and ourselves (54). We have observed further that such thyroid insufficiency produced by feeding neutral fat (cottonseed oil) over long periods of time is accompanied (after 60 days or longer) by hypercholesterolemia and that this increase in the cholesterol content of the blood is directly proportional to the degree of thyroid insufficiency (*i.e.* hyperplasia) (55).

We find that thyroidectomy in rabbits facilitates and accelerates the deposition of cholesterol and the development of experimental atheromata. The reason appears to be that thyroid-deficient animals are less able to oxidize fats and fatty substances (cholesterol) than normals. Marked hypercholesterolemia, cholesterol deposition, and, in turn, consequent atheroma formation result. In direct accord with this is the old and well known clinical observation that thyroid-deficient animals and man are very prone to develop hypercholesterolemia and atheromatosis (56–61, and others).

At this juncture it should be pointed out that since iodine is indispensable for thyroid function the beneficial effects known to follow the prolonged administration of iodides clinically in atheromatosis and atherosclerosis may be dependent upon the influence that such iodine compounds have upon thyroid activity. There is some evidence that the cholesterol content of the blood can be lowered by the administration of thyroid or iodine (62–64). Marine has demonstrated clearly the effects of small (physiological?) doses of iodine upon thyroid hyperplasia. It seems, however, that large or massive doses of iodine may give rise to additional and important reactions. This is the subject of some investigations now being conducted.

Our evidence indicates that sublethal suprarenal insufficiency does not aid in the deposition of cholesterol and the development of experimental atheromata.

The suprarenalectomized rabbits which we used had recovered from the acute insufficiency following the removal of at least one and three-fourths glands and were well compensated during the subsequent experimental period. That they suffered from varying degrees of suprarenal insufficiency was indicated by the fact that they showed at autopsy some lymphoid overgrowth involving both thymus and lymph nodes and very large hyperplastic thyroids, in 3 instances 8 to 10 times the average normal size. Complete double suprarenalectomy was not done in all cases. A fragment of one gland was left behind purposely in 4 of these animals. Thus, we found that rabbits in a state of sublethal suprarenal insufficiency were not more susceptible to the development of atheromata of the aorta following the feeding of lanolin than rabbits with all organs intact. These findings confirm indirectly the observations of Baumann and Holly (65). In 4 in-

stances (1 thyroidectomized and 3 splenectomized) we obtained evidence which suggests that such a state of suprarenal insufficiency may exert an inhibitory influence upon the development of experimental atherosclerosis.

A propos of this it might be mentioned that atherosclerosis is rare in individuals with lymphatism, status thymicolymphaticus, and similar conditions characterized by lymphoid overgrowth. A constitutional anomaly of similar nature seems to result in animals in which a state of sublethal suprarenal insufficiency has been experimentally produced (66).

All this evidence indicates that conditions which are accompanied by long standing hypercholesterolemia cause or facilitate the deposition of cholesterol within the intima of the aorta. This, we find, as have numerous other workers, initiates the formation of experimental atheromata.

In these experiments with lanolin feeding there was no hypertension (67). The blood pressure readings were determined by the method described by Anderson (68). In other words, we find that experimental atheromatosis in rabbits may develop in the presence of normal blood pressure.

However, we do not maintain that prolonged hypertension when present may not alter the arterial wall so that atheromatosis results. The same applies to other mechanical influences such as forces of pulling and dragging, and of wear and tear, the effects of which are seen so frequently in man in later life (Aschoff (69)). Our findings indicate that the deposition of cholesterol within the intima of the aorta initiates the formation of atheromata and any condition (hypercholesterolemia, mechanical forces, etc.) which predisposes to such precipitation thereby predisposes to the development of atheromatosis. This is in agreement with the findings of Aschoff and of Wacker and Hueck.

SUMMARY.

Alimentary hypercholesterolemia acting over a sufficient period of time (in rabbits with all organs intact, 110 days or longer) causes deposition of cholesterol within the intima of the aorta. Deposition of cholesterol within the intima of the aorta initiates the formation of

experimental atheromata. Thyroidectomy, splenectomy, and gonadectomy augment hypercholesterolemia and thereby facilitate and accelerate the development of experimental atheromata of the aorta in rabbits. Sublethal suprarenal insufficiency does not increase the susceptibility of rabbits to the development of such atheromata.

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THE PRODUCTION OF PURPURA BY DERIVATIVES OF PNEUMOCOCCUS.

III. Further Studies on the Nature of the Purpura-Producing Principle.

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INTRODUCTION.

It will be recalled from a previous (1) communication that pneumococcus extract was found to produce hemorrhagic purpura in laboratory animals.[1] The purpura-producing principle resists oxidation and heating to 100°C. for 10 minutes; it is filter-passing; it is present in the fraction of pneumococcus extract obtained by full saturation with $(NH_4)_2SO_4$ after the acetic acid-precipitable substances have been removed. The principle is not associated with type specificity or virulence of the organism and is distinct from the endohemotoxin always present in reduced extracts of Pneumococcus (2). The opinion was expressed at the time that the purpura material was probably a degradation product of pneumococci, since it is present only when cell disintegration accompanied by autolysis has taken place.

Subsequent study (3), established the fact that pneumococcus extract is thrombolytic both *in vivo* and *in vitro*, and that the development of purpura in mice following the injection of pneumococcus extract is associated with an excessive diminution in the number of blood platelets, the greatest decrease usually occurring within 24 hours. In addition the red blood cells also are greatly reduced numerically,

[1] Since our preceding paper, we have seen a reference of Carnot (Carnot, P., *Compt. rend. Soc. biol.*, 1899, li, 927) in which he describes reactions in rabbits to a "pneumococcus toxin." His descriptions indicate the development of purpura in these animals.

but the rate of their disappearance is slower than that of the thrombocytes. Although heat inactivates both the thrombolysin and hemolysin, it has no effect on the purpura-producing property of pneumococcus extract. Adsorption of extract with blood platelets reduces the activity of the thrombolysin, but does not influence the purpura-producing activity in animals.

In the present communication, further studies are reported upon the nature of the purpura-producing principle. Observations are recorded on (1) the immunological reactions, (2) the biological significance and (3) the further chemical fractionation of this substance in pneumococcus extracts.

I. Immunological Reactions of the Purpura-Producing Principle.

(*a*) *Antigenicity of the Purpura Material.*—The purpura-producing substance, fractionated as will be described later, was used as immunizing antigen in rabbits. It was noted that during immunization, purpura lesions which accompanied the earlier injections, were not induced by the succeeding inoculations of the material. After several weeks of immunization, the sera were tested for neutralization of purpura in white mice and for the presence of pneumococcus antibodies. Not only did these sera not possess the power of neutralizing the purpura substance, as is brought out below, but they contained no antibodies for the intact cell or its derivatives. Thus, precipitins were not demonstrated for the purified purpura principle; for the soluble specific substance of the homologous type of Pneumococcus, nor for either extract or protein of the cell. Similarly, it was not possible to show agglutinins for either the "S," encapsulated cell, or the "R," capsule-free cell; nor were the sera able to confer protection upon mice against infection with homologous or heterologous types of Pneumococcus. In this respect, the purpura principle differs markedly from the hemotoxin, since the latter is antigenic (2, 4).

(*b*) *Active Resistance to the Purpura Principle.*—The rabbits employed in the foregoing experiment indicated that notwithstanding the fact that the purpura substance is non-antigenic, an increased resistance may be acquired against purpura. In order to study more thoroughly whether resistance can be exhalted actively, mice were treated with heat-killed suspensions of Pneumococcus, or with

the protein (5), autolysate (1) or extract (6) of the bacterial cells. All the substances are known to stimulate the formation of antibodies (7) and the last two contain the purpura principle. Accordingly, mice received five weekly injections of the particular antigen either subcutaneously or intraperitoneally. 7 to 10 days after the final injection, the mice were tested for increased resistance to purpura.

TABLE I.

Increased Resistance to Purpura Acquired by White Mice Following Repeated Injections of Pneumococcus Extract.

Date of injection	Amount of extract injected	Number of mice injected	Number of mice developing purpura
	cc.		
Apr. 26	0.1	10	10
May 3	0.1	10	10
May 10	0.2	10	8
May 17	0.2	10	2
May 24	0.4	10	0
June 1	0.5	10	0

TABLE II.

The Protective Action of Immune Sera against Purpura in White Mice.

Sera	Number of mice injected	Mice developing purpura	
		Number	Per cent
Controls (no serum)	10	7	70
Normal serum	20	16	80
Antipurpura	10	10	100
Antipneumococcus	5	5	100
Anti-R	15	7	46
Anti-extract	25	13	52
Anti-autolysate	25	21	84
Antiprotein	20	14	70

It was found that repeated injections of pneumococcus vaccine or protein do not increase the tolerance of mice to purpura. Mice so treated developed purpura following the injection of the same amount of extract as was required for the production of purpura in normal animals. Immunization to pneumococcus extract or autolysate, on the other hand, definitely renders mice more resistant to pur-

pura. This fact has been demonstrated satisfactorily a number of times, and a protocol typical of the results (Table I) shows that ten mice were made refractory to purpura by repeated injections of increasing amounts of pneumococcus extracts. It is also of note that the purpura substance no longer causes a diminution in the number of blood platelets or red blood cells in mice whose tolerance to purpura has been raised.

(*c*) *Passive Protection against the Purpura Principle.*—Whether white mice can be passively protected against purpura, was studied by the use of a number of immune rabbit sera prepared by immunization with the purpura principle itself, with "S" and "R" strains of Pneumococcus and with cell extracts, autolysates and the nucleoprotein fraction of the organism. In determining the neutralizing action of the sera against purpura, mixtures of 0.5 cc. of serum and 0.1—0.2 cc. of extract were injected intraperitoneally either immediately on mixing or after incubation together for 1 hour at 37°C. The sera prepared by immunization with the purpura material did not prevent the occurrence of purpura in mice. In no instance, moreover, did any of the sera provide definite passive protection against purpura. The data in Table II show the variation in susceptibility of individual mice and consequently the difficulty of evaluating experiments of this kind. While in the present instance 70 per cent of the normal mice developed purpura, other observations have shown variations from 100 per cent (*cf.* Table I) to 30 per cent of normal mice reactive to a fixed dosage. However, it is sufficiently clear that the sera included in the study did not confer upon normal mice protection against purpura. The lower percentage of animals developing purpura when anti-extract and anti-R sera were utilized, is suggestive of a partial protection, but experience with the purpura substance makes more plausible the interpretation of variation in susceptibility.

The evidence obtained, therefore, is that the purified purpura principle is non-antigenic, in the sense that it produces no demonstrable antibodies. White mice injected repeatedly with substances containing the purpura material acquire an increased tolerance to purpura. On the other hand, the sera of rabbits immunized with the purpura principle, the intact cell or its derivatives confer upon white mice no passive protection against purpura.

II. Biological Nature of the Purpura-Producing Principle.

It was shown in a preceding paper (1) that the purpura substance was found only in those cell derivatives which represented degradation products of pneumococci. The evidence thus far indicates that the substance is not contained as such in the cell, but rather that the purpura principle is a product accompanying cell autolysis. The present experiments substantiate this point of view and confirm the autolytic origin of the purpura material.

Attempts to obtain the purpura substance by dissolution of the cell with bile were repeated, but again the bile solutes were found incapable of producing purpura. Pneumococci were dissolved also by means of sodium oleate, but the resulting solutions did not contain the purpura principle. Curiously enough, incubation of bile solutes to allow digestion by the autolytic enzymes present in the cell did not yield the purpura substance.

Extracts prepared by rapidly freezing and thawing heavy suspensions of live pneumococci are actively purpura-producing, and there is reason to believe that this activity is dependent upon the action of the enzymes of the cell. Since the enzymes of Pneumococcus are inactivated (8, 9) by heating, whereas the purpura principle is resistant to heat (1), it was possible to determine the rôle of the enzymes in the derivation of the purpura substance. Extracts were prepared by freezing and thawing pneumococci previously exposed to 100°C. for 5 minutes. The extracts prepared from heat-killed cells, however, were found to be incapable of producing purpura in white mice. This is further evidence that the purpura principle is not preexistent in the cell.

Avery and Cullen (8, 9) have shown that cell-free filtrates of Pneumococcus contain, among other enzymes, a potent bacteriolytic enzyme which is operative on a substrate of heat-killed pneumococci. It became possible therefore to analyze the relationships of autolytic digestion to the formation of the purpura principle. A solution of active pneumococcus enzymes prepared according to the method of Avery and Cullen was allowed to act on cells killed by heating for 5 minutes at 100°C. After digestion at 37°C., there was distinct lysis of the organisms and cell-free filtrates were obtained after 24, 48,

72 and 96 hours. The filtrates, when injected into white mice, were found to be actively purpura-producing. The enzyme-containing extract, when diluted to the same extent as the filtrates obtained from the digestion experiment, did not produce purpura.

A pneumococcus enzyme solution was inactivated by boiling. It was then allowed to act on heat-killed pneumococci as in the above experiment. No lysis was observed, however, and the filtrates obtained from these tests did not produce purpura. Similar tests conducted with active enzymes in the presence of bile showed that the purpura substance was not demonstrable under these conditions.

The evidence indicates definitely, therefore, that the purpura principle is not a preformed constituent of the cell, but that it is a product of autolysis.

Avery and Cullen (9) have shown that the enzymes of Pneumococcus cause lysis not only of Pneumococcus itself, but also of *Streptococcus viridans*. It was desirable, therefore, to determine whether purpura-producing properties could be obtained from streptococci by digestion with pneumococcus enzymes. Heavy suspensions of *Streptococcus viridans*, killed by heating at 100°C. for 10 minutes, were subjected to the action of pneumococcus enzymes as described in the preceding experiment. Filtrates obtained from the resulting bacteriolysis of streptococcus by pneumococcus enzymes were not capable of producing purpura in white mice.

Purpura is rarely seen in white mice during the course of experimental infection with Pneumococcus. This is possibly due to the fact that the virulent organisms are tremendously invasive and cause death rapidly. Since degraded and avirulent "R" cells yield as potent purpura-producing extracts as their virulent antecedents (1), a study of the disintegration of pneumococci provided a means for determining the occurrence of purpura following the injection of live pneumococci *in vivo*. Small and large amounts of concentrated suspensions of live "R" cells were injected both subcutaneously and intraperitoneally in mice. The majority of the mice survived but in no instance was purpura observed.

In summary, then, it can be said that the purpura-producing principle of Pneumococcus does not exist preformed in the cell. It definitely accompanies autolysis *in vitro* and, under the conditions

stated, purpura was not observed in animals injected with large amounts of live pneumococci.

III. Chemical Nature of the Purpura-Producing Principle.

Previous studies indicated (1) that the purpura substance was precipitated by full saturation with $(NH_4)_2SO_4$ after the nucleoproteins had been previously removed from pneumococcus extract. It was not clear, however, whether the purpura material was albumin or not. In the present study, further fractionation has been accomplished and the purpura principle has been separated from the original precipitate obtained with $(NH_4)_2SO_4$.

In the chemical purification, aqueous extracts of Pneumococcus were employed. Normal acetic acid was added to the extract until no further precipitation occurred. The acid precipitate was removed by centrifugation and the supernatant was withdrawn. The supernatant was heated to maximum coagulation, and the coagulum was whirled down. The materials removed by acid and by heat, as well as the supernatant after removal of both proteins, were studied.

As was previously shown (1) the acetic acid precipitate or nucleoprotein was not purpura-producing. The heat-coagulable proteins were irregular in their action and at best only faintly reactive. Since they were not washed, it seems likely that they were not entirely free from the supernatant. The supernatant remaining after both the acid-precipitable and heat-coagulable proteins had been removed, usually produced marked purpura in mice. The indications are therefore that the purpura principle is not present either in the acid-precipitable or heat-coagulable proteins.

The active purpura-producing supernatant as such gave no qualitative color tests for protein. When it was concentrated to one-tenth volume however, several tests were obtained which aid in identification of the principle. With the biuret test, a pink to purple color was observed. The xanthoproteic test was negative, and Millon's reagent gave a yellow to brown precipitate. A precipitate was obtained with full saturation of $(NH_4)_2SO_4$; the precipitate first appeared at about 60–70 per cent saturation and it increased in intensity up to full saturation. Both picric acid and trichloracetic acid gave precipitates, but in both instances the precipitates vanished

on heating and returned on cooling. Since the concentrated solution, moreover, gave no further coagulation on boiling, it appears that the purpura-producing principle of Pneumococcus is identified with a primary proteose. It cannot be said whether it is actually a proteose or some closely associated substance. The purpura material is present in such small amounts and some is lost during fractionation, so that it becomes a difficult procedure to effect a further separation.

DISCUSSION.

The experiments recorded in this communication disclose that the purpura-producing principle of Pneumococcus does not stimulate the formation of demonstrable antibodies. The serum of animals immunized with either the purpura principle itself, with pneumococcus cells or with extract, autolysate or nucleoprotein of the bacteria does not afford white mice passive protection against purpura. In contrast to this fact, however, white mice and rabbits acquire an increased tolerance to purpura following successive injections of materials containing the purpura substance. It seems likely, therefore, that the "active immunity" in this instance is really an increased resistance to the toxic agent. This is supported by two facts: (1) the purpura-producing principle is not antigenic, and (2) passive protection against purpura is not transferred to normal mice by the serum of resistant animals.

The experiments on the biological nature of the purpura principle furnish direct evidence that it accompanies autolysis and that it is most probably a degradation product. It was not obtained as a preformed constituent of the cell, since, in the absence of enzyme action, cytolysis of live or dead cells did not yield the purpura substance. Dissolution of the live cell by bile or sodium oleate, neither of which inhibits the activity of the purpura principle when once formed, did not furnish the purpura material. Moreover, the presence of bile has been observed to inhibit definitely the formation of the purpura substance. In fact Jobling and Strouse have shown that a number of unsaturated fatty acids, including sodium oleate, actually do inhibit the action of proteases. Since bile does contain unsaturated fatty acid, there is some support for the belief that bile inhibits the digestive processes which give rise to the purpura prin-

ciple. Freezing and thawing heat-killed organisms in the absence of active enzymes did not liberate the purpura principle. On the other hand, the digestion of dead cells by active enzymes of Pneumococcus gave filtrates which were actively purpura-producing. In other words, the purpura substance appears to be an autolytic derivative of Pneumococcus.

Further fractionation of the purpura material indicates that it is closely related to a primary proteose. It has been definitely separated from both the acid-precipitable and heat-coagulable proteins of pneumococcus extract, but a more exact identification cannot be stated at the present time. In general proteoses have been found to be non-antigenic (10) although the observations of some investigators notably those of Fink (11) indicate that proteoses may be antigenic. That proteoses, moreover, may be toxic has been demonstrated by a number of investigators (12).

CONCLUSIONS.

1. The purpura-producing principle of Pneumococcus is non-antigenic in the sense that it does not stimulate the formation of antibodies.

2. White mice acquire an increased resistance to purpura as a result of repeated injections of toxic doses of the purpura substance.

3. The serum of rabbits immunized with the purified purpura principle, with "S" and "R" strains of Pneumococcus or with cell extracts, autolysates or the nucleoprotein fraction of the organism does not confer upon white mice protection against purpura.

4. The purpura principle does not exist preformed in the cell, but is rather an autolytic derivative; since it is formed only when pneumococci undergo autolysis, and it is not found when the autolytic ferments are inactivated.

5. The purpura substance is associated with the proteose fraction of active pneumococcus extracts.

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THE RELATION OF THE RETICULO-ENDOTHELIAL SYSTEM TO THE FORMATION OF AMYLOID.

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Plates 20 to 22.

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In a recent article (1) Domagk states that he succeeded in producing amyloid in the spleen and liver of normal mice within 10 minutes by intravenous injection of a large quantity of living cocci; furthermore, that he could produce amyloid within 2 minutes in the spleen and liver of a mouse which had several injections of dead cocci previous to the final intravenous injection of living cocci. He also described marked changes in the reticulo-endothelial cells, and finally drew the conclusion that amyloid appears at first in the neighborhood of these phagocytic cells. This substance which he thought to be amyloid did not react to any of the specific stains (gentian violet or iodine), but showed metachromasia when stained with cresyl violet or Giemsa.

The Influence of the Injection of Dead and Living Bacteria upon the Formation of Amyloid.

Since vital staining by the intravenous injection of Congo red has proved in many experiments to be a most delicate and reliable, as well as specific, method for demonstrating even the earliest traces of amyloid (2), experiments were undertaken in order to define the nature of the substance produced in Domagk's experiments.

Each of a series of 10 mice was injected with 0.2 cc. of dead streptococci in the gluteal region four times at intervals of 2 days. Following these injections 0.5 cc. of living streptococci was injected intravenously into each animal. In another series of 10 mice 0.5 cc. of living streptococci was injected intravenously without any previous injections of dead bacteria. Corresponding animals of each group were killed 15 minutes, 30 minutes, 1 hour, 2 hours, 24 hours, 2 days, 3 days and 7 days after injection. Each of these animals received 0.5–0.7 cc. of 1 per cent solution of Congo red intravenously shortly before death.

None of the 20 animals showed even the slightest trace of amyloid in any of the organs, although many of them showed marked lesions in spleen and liver corresponding to those described by Domagk. The changes in the reticulo-endothelial cells are very marked and consist of swelling and vacuolation. These cells contain bacteria for some time after the injections and later on become distended with red blood cells and cellular debris.

These experiments obviously shed no light upon the relation of the reticulo-endothelial cells to amyloid formation since there was no evidence that amyloid was formed. The idea, however, that reticulo-endothelial cells could in some way be responsible for the formation of amyloid is very tempting in many respects. It would at once explain many morphological pictures seen in cases of experimental amyloidosis, the interpretation of which appears to be rather forced so far.

The Relation of the Reticulo-Endothelial Cells to the Site of Formation of Amyloid.

It is surprising indeed to find the phagocytic reticulo-endothelial cells of spleen, liver, kidney, adrenal and intestines located exactly in these places where amyloid is constantly seen. After one or more intravenous injections of 0.2 cc. of India ink in a mouse, the reticulo-endothelial cells filled with ink particles are seen forming black rings in the tissue around all the follicles of the spleen, while there is comparatively little ink phagocytized in other parts of this organ (Figs. 1 and 2). In the liver one finds ink in cells, located beneath the endothelium of the periportal vessels, and sometimes in the endothelial cells themselves. The many phagocytic cells in the capillaries and the Kupffer cells are loaded with ink particles and often appear as dark, solid black spots, their nuclei being unrecognizable. There are only a few of these cells seen around the central vessels (Figs. 3 and 4). In the kidney the ink is found in cells of the capillaries of the glomeruli, and in the medulla between tubules (Figs. 5 and 6); in the adrenals in phagocytic cells of the capillaries between the columns of the cells in the cortex; in the intestines most of the ink is seen in the stroma of the villi, especially at their tips, and also in the capillaries at their bases (Figs. 7 and 8). All of these are the very places where amyloid is invariably and typically found when present.

Another point in favor of the theory that the reticulo-endothelial

cells may play an active part in the formation of amyloid is the solitary appearance of small, isolated patches of amyloid in all of the organs concerned. This phenomenon can, of course, only be observed in very early stages, and is even then rarely seen in the spleen, which fact is responsible for the idea that amyloid appears at once in masses around the follicles. This is not true, for here, as elsewhere, there are first formed small isolated droplets of amyloid which anastomose later and form the solid ring which is so typical of this organ (Fig. 9).

In the liver the earliest form of amyloid is found in the form of small solitary patches in the tissue surrounding the periportal vessels and in the intermediate zone of the lobules (Fig. 4). These isolated areas soon unite, thus forming a complete layer of amyloid around the vessels. At the same time solid, anastomosing strands of this material are found in the adjoining parts of the liver lobules. The tissue around the central vessels is much less affected but not completely exempt. Some of the patches of amyloid appear to be located in the lumen of the capillaries, often completely surrounded by endothelial cells; this fact is mentioned by many authors and all sorts of explanations, which on the whole are rather unsatisfactory, have been made for this remarkable occurrence which seems to contradict any present theory of the mechanism of amyloid formation.

In the glomeruli and in the tissue of the papillæ of the kidneys, between the columns of cortical cells in the adrenals, in the stroma of the villi of the intestine, as well as in the tissue at their bases, one sees the earliest stage of amyloid represented by small isolated patches which later on form anastomoses which imitate the whole framework (Figs. 6 and 8). The same principle governs the appearance of amyloid in all these organs.

The solitary appearance of small patches of amyloid in different parts of different organs suggests its formation on the very spot where it is located rather than precipitation of a substance circulating in the blood stream. The assumption that amyloid is formed in some way by activities of specific cells would not be contradictory of morphological facts. It cannot, however, be too strongly emphasized that only studies of the early stages of amyloidosis are of any value as far as its genesis is concerned. In this sense the study of

chronic, even advanced cases, would be of about the same value as those of scars in questions of tuberculosis or syphilis.

Delay in Appearance of Amyloid Following Attempt at Blockage of Reticulo-Endothelial System with India Ink.

In order to demonstrate possible corelations between reticulo-endothelial cells and amyloid, 15 of a series of 30 mice (Series 1-b, Table I) were injected intravenously with India ink, and amyloid was then produced by subcutaneous injections of 0.5 cc. of 5 per cent nutrose as used by Kuczynski (3). An injection of 0.2 cc. of India ink was given 1 day before the first injection of nutrose. If such a mouse is killed 1 day after the injection, practically all the reticulo-endothelial cells are found to be loaded with ink particles. Later on one often finds cells, which have to be called reticulo-endothelial cells as far as their morphology and position go, without any ink particles in their protoplasm. This is not surprising since many of the ink-containing cells die and are replaced by new cells, most of which certainly contain no ink. The majority of the reticulo-endothelial cells, however, still show ink particles 2 months after a single injection. In general one can assume that most of the cells containing ink were present at the time of the injection.

On the same day the other half of the series (Series 1-a, Table I) were injected with nutrose only, for control purposes. At certain intervals animals were simultaneously killed from both series. The organs of mice which died spontaneously were fixed in Zenker, formalin and alcohol, and sections of the various organs stained for amyloid with Congo red (4) and gentian violet. All the other animals were injected intravenously with 0.5–0.7 cc. of 1 per cent Congo red at least ½ hour before death in order to mark out the amyloid vitally. Their organs were fixed in saturated corrosive sublimate.

In the mice injected with nutrose alone (Series 1-a, Table I) amyloid was found after about 30 daily injections, while all the previously killed animals showed none. The corresponding animals (Series 1-b, Table I) injected with India ink 30 days previously did not have any amyloid. These results suggested that the appearance of amyloid was retarded in those animals whose reticulo-endothelial cells were loaded with ink, but upon repeating the experiment (Series 2-a and b, Table I) amyloid was found to appear at about the same time in the mice receiving India ink and nutrose, as in those receiving nutrose alone.

In the attempt to settle this question of the possibility of influencing the time of appearance of amyloid by blocking the reticulo-endothelial cells with India ink, four more similar series of animals were studied. Realizing that only one injection of ink was perhaps not enough to block the reticulo-endothelial system very extensively and that this fact might have been responsible for the indecisiveness of

the second experiment, those mice receiving ink of each of these four latter series were injected three to ten times during the course of the experiment.

TABLE I.

The Relation of Attempted Blockage of Reticulo-Endothelial System by Injection of India Ink to the Time of Appearance of Amyloid.

Repeated subcutaneous injections of nutrose 0.5 cc., 5 per cent solution 6 times a wk.				Intravenous injection of India ink before and during the experiment. Repeated subcutaneous injection of nutrose 0.5 cc., 5 per cent solution 6 times a wk.				
Series	Duration of experiment in days	Numbers of mice	Amyloid	Series	Duration of experiment in days	Numbers of mice	No. of intravenous injections of India ink	Amyloid
1-a	30	1–13	−	1-b	30	1–13	1	−
	31	14	+		31	14	1	−
	32	15*	−		36	15	1	−
2-a	13	1–14	−	2-b	38	1–13	1	−
	45	15	+		44	14	1	+
					52	15	1	+
3-a	36	1–13	−	3-b	35	1–12	1–2	−
	38	14	+		37	13	3	−
	42	15	+		41	14	3	−
					50	15	3	+
4-a	6	1–13	−	4-b	14	1–12	1	−
	22	14	−		28	13	2	−
	25	15	+		38	14	3	−
					47	15	5	+
5-a	34	1–13	−	5-b	30	1–13	1–4	−
	36	14	−		36	14	5	−
	42	15	+		42	15	5	−
6-a	18	1–11	−	6-b	18	1–13	1–3	−
	42	12	−		42	14	7	−
	47	13	+		53	15	10	−
	48	14	+					
	53	15	+					

* Received one intravenous injection of India ink 15 days before death.

In each of these four series amyloid appeared later in those animals whose reticulo-endothelial cells were filled with India ink than in the controls receiving nutrose alone (see Table I).

Morphological Relationship of Newly Formed Amyloid and Ink-Containing Reticulo-Endothelial Cells.

In studying the morphological relations of reticulo-endothelial cells and amyloid, especially in differentiating reticulo-endothelial cells present before the appearance of amyloid and those formed during and after its formation, only mice of Series 1 and 2 receiving one injection of ink, were used. The findings are constant but difficult to interpret. The clear-cut structure of the liver makes it easier to study the relationship between reticulo-endothelial cells and amyloid in this organ than in spleen and kidney; therefore, most of the observations were made in the liver. An average periportal field shows the following picture. A ring of amyloid is found between the endothelium of the vessels and the liver cells. Cells containing ink granules may be seen lining the lumen but are more often found outside the zone of amyloid, less frequently within. These cells appear to be intact and their nuclei are present. Embedded in the substance of the amyloid one occasionally sees isolated small particles of ink, having no longer any obvious relationship with living cells; but often there is no trace of ink in relatively large solid masses of amyloid (Fig. 10). In earlier stages the localized patches of amyloid around the periportal vessels are often seen to be completely surrounded by ink particles of various sizes, while there is no trace of ink in the amyloid itself (Fig. 11). In the midzonal areas of the lobules the relations between cells containing ink particles and patches of amyloid are manifold. The area of amyloid may be covered on one or the other side by cells containing ink (Fig. 12). These cells, in addition to other cells which do not contain ink, may also be found completely surrounding the amyloid (Fig. 13). Sometimes, but not frequently, cells or remains of cells with ink granules lie in the center of a small, solid area of amyloid, which is arranged in concentric layers and in turn is separated from the liver cells by ink-containing reticulo-endothelial cells (Fig. 12). Some of these ink granules in the periphery of an area of amyloid do not seem to be located any longer in the cells (Fig. 14). It appears as though these particles represent remains of cells which no longer exist. Not infrequently separate contiguous, crystalline patches of amyloid, completely

surrounded by liver cells, were found. Cells which contain ink granules separate these patches from the liver cells and there are also a few small particles of ink between them (Fig. 15).

In the spleen the cells which contain ink are widely scattered throughout the areas of amyloid, especially in the periphery of the zone of amyloid surrounding the follicles (Fig. 16). Isolated phagocytic cells are frequently found completely embedded in amyloid and free particles of ink are often enough seen lying in this substance (Fig. 17). Solitary patches of amyloid in the pulp are usually surrounded by cells which contain ink, sometimes only by ink granules (Fig. 18).

The interpretation of these findings in the liver and spleen appears difficult. One thing seems to be certain, that the reticulo-endothelial cells as such are not primarily transformed into amyloid. Were this true one would expect to find amyloid constantly containing large numbers of ink granules; this, however, is not the case. The granules which are actually seen included in masses of amyloid all appear to be the remains of cells passively involved in this process.

For pictures where the patches of amyloid are completely surrounded by cells containing ink, it is difficult to offer any other explanation except that these patches are situated in capillaries; otherwise how could amyloid, were its position outside the wall of capillaries, be surrounded by reticulo-endothelial cells which contain ink injected 6 weeks previously. Some of these cells may be phagocytic wandering cells, but most of them are certainly reticulo-endothelial cells as far as their morphology goes.

Only occasionally is there a suggestion that amyloid is situated within reticulo-endothelial cells. Fig. 19 shows two of these cells which seem to be filled with both ink and amyloid. Such appearances are very rarely seen.

Although one cannot always clearly interpret these various morphological relationships of amyloid, reticulo-endothelial cells and ink, none of them are contradictory to the idea that amyloid may in some way be formed through the activity of reticulo-endothelial cells. On the other hand, however, certain pictures (Figs. 13–15) demand this interpretation.

Injection of India Ink in Mice Already Showing Amyloidosis.

If one assumes that the reticulo-endothelial cells form amyloid, the question arises whether such an unusual process does not alter the cells greatly, or whether this substance is perhaps only formed by damaged and altered cells.

Should the reticulo-endothelial cells be involved only secondarily one would expect still to find many of these cells, which are yet phagocytic, in areas of already developed amyloid. There should also be ink-containing cells found in the periphery of follicles in the spleen and around the periportal fields of the liver after an injection of India ink in an animal having amyloidosis. One should expect to find their number decreased but there would be definite evidence of the presence of many of them which would take up the ink in a way similar to that of a normal animal. A mouse having received about 50 subcutaneous injections of nutrose was injected for the first time with 0.2 cc. of India ink intravenously 1 day before it was killed, and received $\frac{1}{2}$ cc. of 1 per cent Congo red $\frac{1}{2}$ hour before its death.

The sections of the organs of this animal show striking pictures. There are huge masses of vitally stained amyloid around the follicles in the spleen, and there are amyloidal rings around the periportal vessels of the liver, and some patches of amyloid in the intermediate zones.

There are almost no reticulo-endothelial cells containing ink granules around the follicles except in places where there is no amyloid, or where the mantle of amyloid around the follicles is not complete. Only a few of these cells are seen in large areas of amyloid (Fig. 20).

In the liver there are numerous cells loaded with ink far away from the periportal fields, while they are practically missing in a broad zone around the vessels themselves, where amyloid is most marked (Fig. 21). Identical pictures were seen in organs of mice injected three to five times subcutaneously with 1 cc. of a 1 per cent solution of trypan blue after the development of amyloid.

The striking absence of phagocytic cells in the areas where amyloid is most abundant must mean that the reticulo-endothelial cells of these areas have been destroyed or have been markedly altered physiologically. Were such pictures exceptional one might think that ink had

not reached the reticulo-endothelial cells due to obstruction of the periportal capillaries by amyloid and that ink reached only the reticulo-endothelial cells in the central portion of the lobule through collateral circulation from adjacent lobules. This conception of vascular obstruction is hardly tenable, however, for every capillary throughout the liver shows the usual amount of reticulo-endothelial cells containing ink particles. The additional fact that the amyloid throughout the entire lobule is brilliantly stained vitally by intravenous injections of Congo red also strongly speaks against a vascular obstruction in these areas.

Evidence of destruction of reticulo-endothelial cells is commonly found in cases of progressing amyloidosis in all the involved organs, and by the observations just described the idea of the direct connection of these perishing cells with the formation of amyloid is strongly supported, but there is no evidence that the cells as such are directly and primarily converted into amyloid.

DISCUSSION.

In this paper facts have been assembled which speak in favor of the theory that amyloid is actively formed by specific phagocytic cells (reticulo-endothelial cells) in the place where it appears. It must be said that all of the material collected does not furnish conclusive proof of this theory, but puts forward facts which suggest it rather strongly.

It is remarkable indeed to find most of the reticulo-endothelial cells in the organs in question in the very places where amyloid lies. The objection of having phagocytic cells in numbers in other organs also where amyloid is not commonly found, for instance in the lungs, must not necessarily overthrow this theory. Having phagocytic properties in common with those of the lungs, the reticulo-endothelial cells of spleen, liver, kidney, intestine, adrenal and lymph glands probably have many other special functions besides, each group adapting itself to the function of the organ in which it is located. This special functional relationship may be the explanation why the reticulo-endothelial cells of spleen, liver and kidney should be more concerned than those of other organs in the formation of amyloid.

The solitary patchy appearance of young amyloid seems to indicate

its formation right on the spot where it appears rather than a precipitation of a substance circulating in the blood stream. That it appears in this form just at the very place where phagocytic cells are found in great number is also in favor of the theory that it might have been formed actively by specific cells. The presence of masses of amyloid in the lumen of capillaries of the liver, surrounded completely by Kupffer cells, could only be explained in this way.

The presence of two or more patches of amyloid lying independently but close together in the lumen of one capillary seems to indicate the mechanism by which continuous streaks of this material are formed. This is the form in which amyloid is seen in advanced cases.

It seems much easier to explain the endothelial covering of amyloid in chronic cases, which makes the amyloid appear to be outside the lumen, by a new growth of endothelial cells making an effort to preserve the smooth lining of the capillary than to interpret the presence of endothelial cells around the amyloid by a new growth of endothelium between amyloid and liver cells. The latter would mean a complete reorganization of the capillary system.

There are signs of severe damage and disintegration of reticulo-endothelial cells, during the process of formation of amyloid, as seen by direct observation and by the absence of cells which phagocytize India ink in the areas of newly formed amyloid. The presence of amyloid itself is a manifestation of such an unusual unphysiological condition that it is not difficult to believe that the material is formed by reticulo-endothelial cells, in an abnormal state.

The reticulo-endothelial cells are also secondarily involved in this process; they become partly and completely surrounded by amyloid and are often found embedded in this substance. Finally the cells die, leaving only their phagocytized granules in their places. It is impossible to say what becomes of their cell substances, whether they turn into amyloid or whether they are simply replaced by this substance.

The results of the experiments in which the attempt was made to block the reticulo-endothelial system with India ink have limited value for two reasons. In the first place it is impossible to block these cells completely; secondly there is a considerable irregularity in the time necessary for the experimental production of amyloid follow-

ing even standardized injections of nutrose. But since the attempted blockage of the reticulo-endothelial cells did result in marked delay of the appearance of amyloid, this fact, though itself inconclusive, at least supports the other observations leading to the idea that these cells are concerned in the formation of amyloid.

CONCLUSIONS.

The facts presented suggest strongly that reticulo-endothelial cells are actively concerned in the formation of amyloid. Points in favor of this theory are as follows:

1. The appearance of amyloid in places where reticulo-endothelial cells are normally present, sometimes in very large number.
2. The formation of early amyloid in the small solitary patches, which suggests its local formation.
3. The occurrence of solitary patches of amyloid apparently located within the capillaries of the liver.
4. The manifold relations between reticulo-endothelial cells marked out by phagocytized ink granules, loose ink particles and amyloid, described in the text.
5. The impossibility of demonstrating reticulo-endothelial cells in areas of forming amyloid by intravenous injections of India ink.
6. The delayed appearance of amyloid in animals after blockage of the reticulo-endothelial cells by repeated intravenous injections of India ink.

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EXPLANATION OF PLATES.

PLATE 20.

FIG. 1. Follicles of spleen. Reticulo-endothelial cells containing phagocytized ink particles around follicles. Three intravenous injections of 0.2 cc. of India ink. Unstained section. × about 75.

FIG. 2. Follicles of spleen. Vitally stained amyloid around follicles. Unstained section. × about 75.

FIG. 3. Peripheral vein of liver. Reticulo-endothelial cells with phagocytized ink particles beneath the epithelial lining and in capillaries. Ink granules in endothelium of vein. One intravenous injection of 0.2 cc. of India ink. Unstained section. × about 110.

FIG. 4. Peripheral vein of liver. Vitally stained solitary patches of amyloid in tissues around the vein and in the intermediate zone of liver lobule. Unstained section. × about 110.

FIG. 5. Cortex of kidney. Phagocytized ink particles in reticulo-endothelial cells of glomeruli. One intravenous injection of India ink. Unstained section. × about 110.

FIG. 6. Cortex of kidney. Vitally stained patches of amyloid in glomeruli. Unstained section. × about 110.

FIG. 7. Intestine. Reticulo-endothelial cells containing ink particles in tissues of villi. Three intravenous injections of India ink. Unstained section. × about 75.

FIG. 8. Intestine. Vitally stained amyloid in stroma of villi. Unstained section. × about 75.

FIG. 9. Spleen. Vitally stained patches of amyloid around follicles and in pulp of spleen. These patches are seen to become confluent in a semicircular area around the follicles. Unstained section. × about 75.

PLATE 21.

FIG. 10. Peripheral vein of liver in the center; the light zone around the endothelial lining represents amyloid. Patches of amyloid in the intermediate zone of lobule. Ink particles are found in endothelium, in reticulo-endothelial cells around the amyloidal ring, embedded in amyloid and in capillaries of the liver lobule. The solitary patches of amyloid are partly or completely surrounded by these cells. Fine ink granules in amyloidal zone around the vein. Amyloid vitally stained with Congo red; tissue stained with iron-hematoxylin. × about 345. Mouse 14. Series 2-b.

FIG. 11. Part of the wall of a periportal vein of liver. Small area of amyloid (*a*) in wall of vein, surrounded by loose ink particles. Amyloid vitally stained with Congo red; tissue stained with iron-hematoxylin. × about 650. Mouse 15. Series 2-b.

FIG. 12. Intermediate zone of liver lobule. Solitary and confluent patches of amyloid. Reticulo-endothelial cells marked out by ink are found around these patches, lining them partially or completely. Ink-containing cells and fragments in the centers of two patches which show a concentrical arrangement (*a*). Fragments of cells and ink granules around small solitary patches of amyloid (*b*). Amyloid vitally stained with Congo red; tissue stained with iron-hematoxylin. × about 345. Mouse 14. Series 2-b.

FIG. 13. Intermediate zone of liver lobule. Solitary patch of amyloid showing outspoken crystalline structure. Reticulo-endothelial cells with phagocytized ink particles surrounding the amyloid completely. Amyloid vitally stained with Congo red; tissue stained with iron-hematoxylin. × about 650. Mouse 14. Series 2-b.

FIG. 14. Intermediate zone of liver lobule. Solitary patch of amyloid showing outspoken crystalline structure. The amyloid is surrounded by reticulo-endothelial cells containing ink and by ink granules. Amyloid vitally stained with Congo red; tissue stained with iron-hematoxylin. × about 650. Mouse 14. Series 2-b.

FIG. 15. Intermediate zone of liver lobule. Three solitary patches of amyloid all showing crystalline structure. *a* and *b* are contiguous, but not confluent. Reticulo-endothelial cells containing ink around *a* and *b*. Fine ink particles between *a* and *b*. Cells with and without phagocytized ink particles around *c*. Amyloid vitally stained with Congo red; tissue stained with iron-hematoxylin. × about 650. Mouse 14. Series 2-b.

PLATE 22.

FIG. 16. Follicle of spleen. *a*, central artery. Zone of amyloid around the follicle. Reticulo-endothelial cells containing ink are scattered throughout the amyloid, but are more frequently seen in the periphery of the amyloidal zone and adjacent tissue. Isolated ink-containing cells and loose ink granules embedded in substance of amyloid. Amyloid vitally stained with Congo red; tissue stained with iron-hematoxylin. × about 190. Mouse 14. Series 2-b.

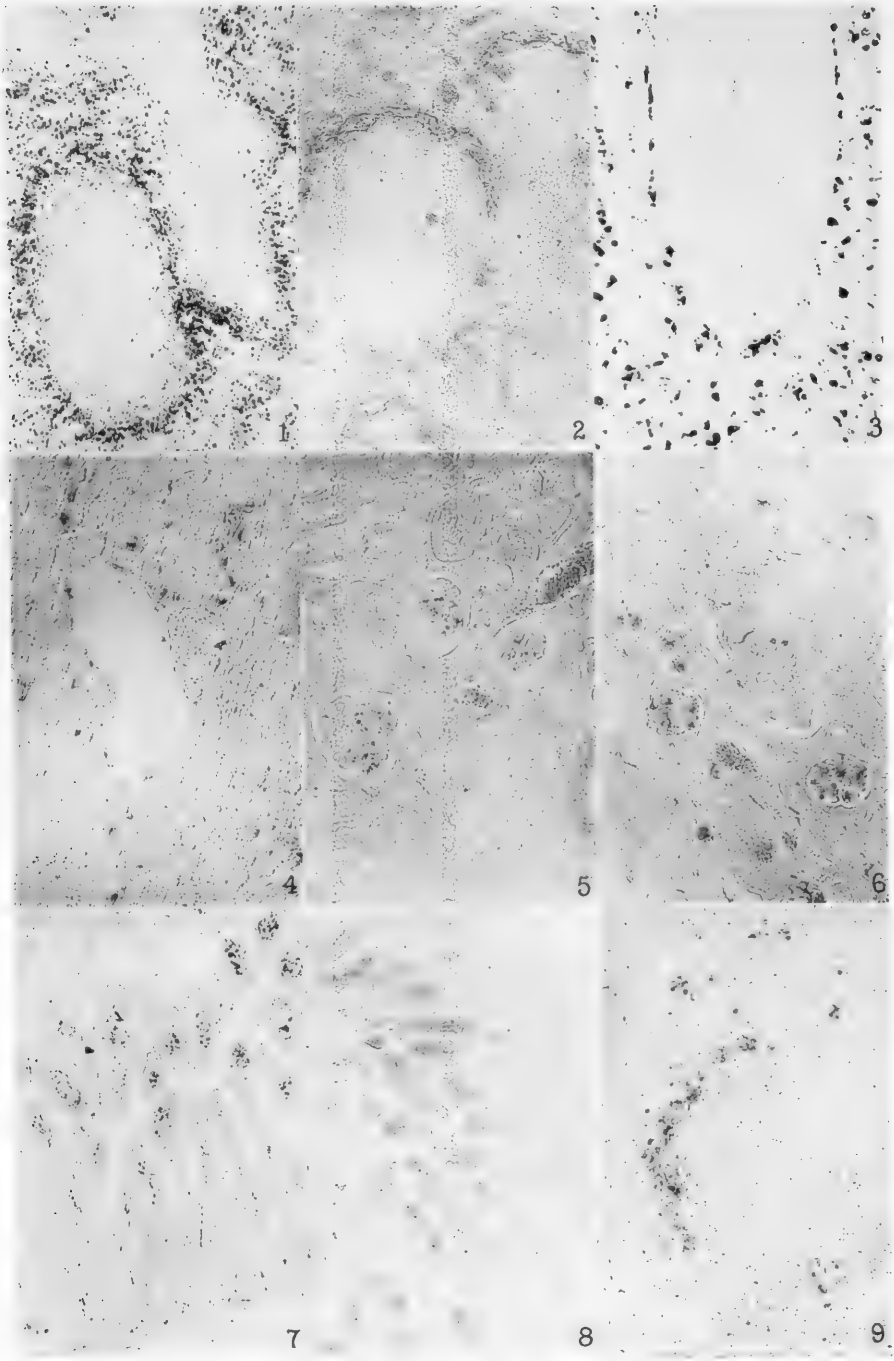
FIG. 17. High power magnification of one part of Fig. 16. Malpighian body (central artery *a*) surrounded by amyloid (unstained). Isolated cells, some containing phagocytized ink particles embedded in amyloid. Also fine ink granules in substance of amyloid. × about 345.

FIG. 18. Splenic pulp. The large light areas are solitary patches of amyloid in tissue of pulp surrounded by reticulo-endothelial cells containing ink and also by ink granules. Amyloid vitally stained with Congo red; tissue stained with iron-hematoxylin. × about 345. Mouse 15. Series 2-b.

FIG. 19. Intermediate zone of liver lobule. Two small solitary patches of amyloid; *a*, almost surrounded by a solid black zone of ink; *b*, enclosed by ink particles which show a bipolar arrangement. Amyloid vitally stained with Congo red; tissue stained with iron-hematoxylin. × about 345. Mouse 14. Series 2-b.

FIG. 20. Follicles of spleen partially surrounded by large masses of vitally stained amyloid. 0.2 cc. of India ink was injected 1 day before death. No reticulo-endothelial cells containing ink around follicles except in area *a* where the amyloidal zone around the follicle is not complete. (Compare with Fig. 1.) Unstained section. × about 75.

FIG. 21. Two periportal vessels of liver. Vitally stained rings of amyloid around vessels, patches of amyloid in intermediate zone. 0.2 cc. of India ink was injected 1 day before death. No reticulo-endothelial cells in tissue around both vessels, no ink granules in endothelium. The capillaries show the usual amount of phagocytic cells loaded with ink. (Compare with Fig. 3.) Unstained section. × about 115.



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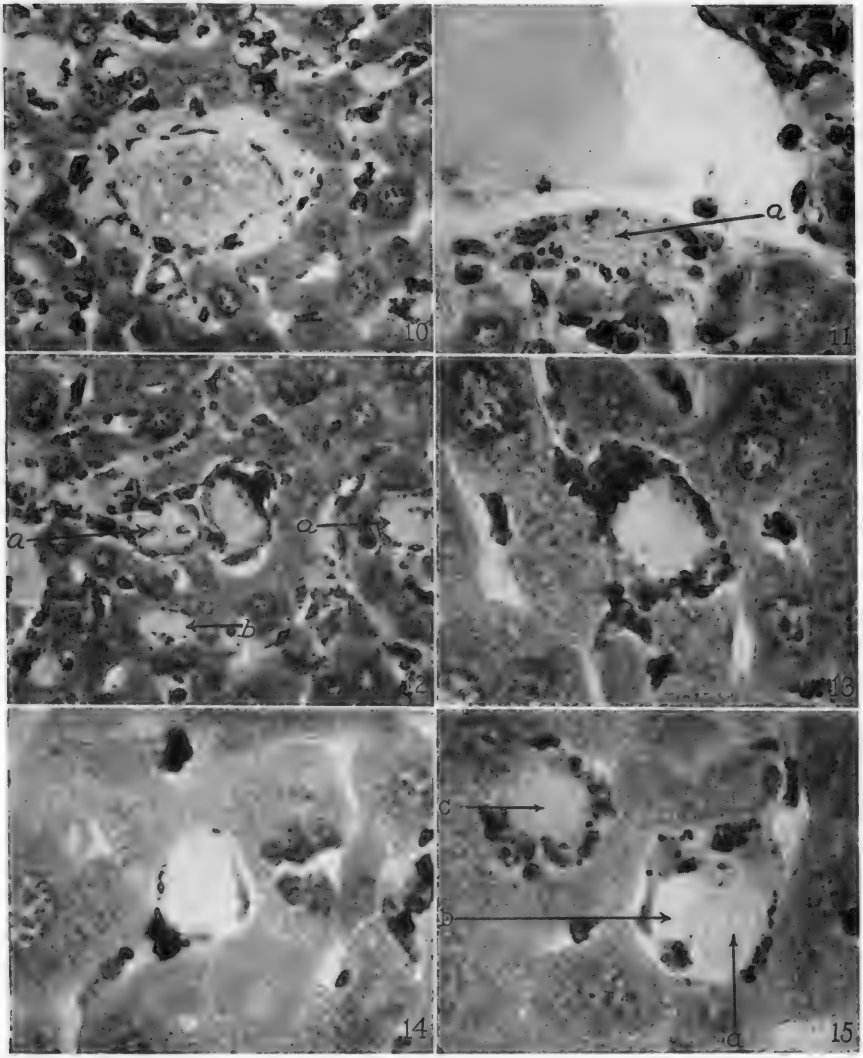

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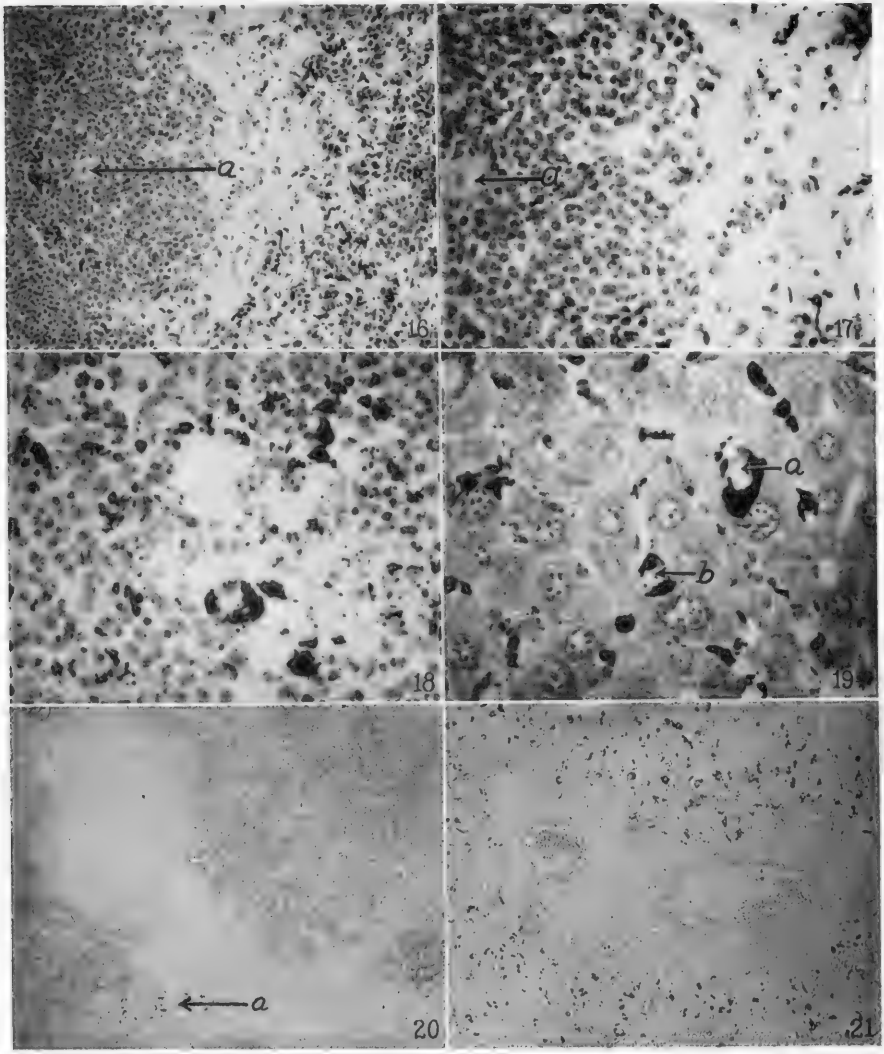

(Smetana: Reticulo-endothelial cells and amyloid.)

CHANGES IN CARBON DIOXIDE TENSION AND HYDROGEN ION CONCENTRATION OF THE BLOOD FOLLOWING MULTIPLE PULMONARY EMBOLISM.

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INTRODUCTION.

In an effort to explain the cause of the extraordinarily accelerated respirations which result from multiple experimental embolism of the arterioles and capillaries of the lungs (1) it seemed important to observe what changes occurred in the blood in respect to its carbon dioxide tension and hydrogen ion concentration. The relative importance of these two factors in the control of respiration has been much discussed in the literature of late years, and opinion has emphasized first one, then the other. It is probable that both the pressure of carbon dioxide and the hydrogen ion concentration of the blood (or of the respiratory center itself) play an important rôle in the chemical regulation of respiration. The characteristic breathing of acidosis, due to the retention of non-volatile acids, such as is seen in diabetic coma, is hyperpnea, or a deep, labored, slow type of breathing. This, too, is the character of respiration which results from inhalation of gas mixtures rich in carbon dioxide (5 to 10 per cent). As far as we know, rapid and shallow breathing has not been definitely related to a retention of carbon dioxide, nor to an increase in the concentration of hydrogen ions. In at least one clinical condition (bronchopneumonia) (2), however, an increased content of carbon dioxide in the blood has been described, and we know that in this condition the respiratory rate may be elevated. That the accelerated respirations which we were attempting to explain resulted from an increased carbon dioxide tension or a fall in pH seemed unlikely from the outset. Still we considered it necessary to make actual measurements of these changes

in relation to the changes in respirations before ruling them out as of importance. Especially was this true in view of the observation made by Dunn (3) that the inhalation of 10 per cent carbon dioxide by goats, breathing rapidly as the result of multiple pulmonary emboli, caused a reduction of the respiratory rate. In the light of our own observations, it seems probable that the cause of this reduction was probably an increased depth of respiration stimulated by the carbon dioxide in the inspired air. Such an increased depth would lead to an increase in alveolar oxygen tension and hence a decrease in the anoxemia. Anoxemia has been shown (1) to be a contributing factor to the rapid and shallow breathing resulting from multiple embolism of the pulmonary arterioles and capillaries, though not its cause.

EXPERIMENTAL.

The experimental procedure was a simple one. Dogs were anesthetized by the intravenous injection of barbital-sodium. A cannula was inserted into the left femoral artery, from which samples of blood were drawn for analysis, and into the right external jugular vein into which suspensions of potato starch cells were slowly injected. The blood was drawn without exposure to air and kept in sampling tubes over mercury. Analyses of carbon dioxide content were made in duplicate on the separated plasma or serum by the method of Van Slyke and Neill (4). The pH was determined on serum by the colorimetric method of Hastings and Sendroy (5). From these analytical data and from the following formula (6) the partial pressure of carbon dioxide expressed in mm. Hg was calculated, assuming pk' to be 6.115:

$$pCO_2 = \frac{[CO_2]}{0.031 \times (1 + 10^{pH - 6.115})}$$

where CO_2 content is given in terms of millimols per liter. In some experiments the tidal air and minute volume of pulmonary ventilation were measured by securing a tightly fitting mask to the dog's muzzle and collecting the expired air by means of flutter valves in a Tissot spirometer. In other experiments the respiratory rate was simply counted by observing the animal's thoracic movements. In most of the experiments, anoxemia was prevented by permitting the animal to breathe oxygen. It was soon learned that the rapid and shallow breathing which comes on after a certain volume of starch suspension has been injected, is usually associated with an increase in the carbon dioxide tension of the plasma and an accompanying fall in pH. But subsequent observations revealed the fact that these changes do not necessarily occur, and sometimes follow rather than precede, the onset of abnormally accelerated respirations.

To test the effect of the experimental conditions *per se*, without starch injection, on the carbon dioxide tension and hydrogen ion concentration, these measurements were made on a dog at approximately hourly intervals over a period of 3 hours. The results showed a fall in pCO_2 and a rise in pH, or a change in the opposite direction from that usually encountered following embolism. These facts are shown in Table I.

In the following three experiments (Nos. 4, 7 and 9) the accelerated respirations following starch embolism were accompanied by a marked

TABLE I.

Experiment 6. Effect of Barbital-Sodium Anesthesia on pCO_2 and pH of Dog's Blood and on Respiratory Rate.

Time	Respiratory rate per min.	CO_2 content	pCO_2	pH
		mM	*mm.Hg*	
2.47	38	23.37	46.20	7.30
4.00	34	22.02	38.20	7.36
5.00	36	21.67	36.80	7.37

TABLE II.

Experiment 4. Effect of Intravenous Starch Suspension Injection on Respiratory Rate, CO_2 Tension and pH of the Blood.

Time	Procedure	Respiratory rate per min.	CO_2 content	pCO_2	pH
			mM	*mm.Hg*	
4.04		16	21.17	47.50	7.23
4.12–4.32	Injection of starch suspension				
4.33		52			
4.35		51	22.28	60.68	7.15

increase in CO_2 tension (from 5 to 13 mm.) and a definite fall in pH (from 0.07 to 0.09). In each instance characteristic rapid and shallow breathing occurred, the rate increasing from an average of 18 to the minute to an average of 46, and the depth decreasing accordingly. The experimental data are brought out in Tables II to IV. It is to be noted that the actual change in CO_2 content expressed in millimols may be slight when the change in CO_2 tension is considerable.

Examination of Table III will bring out the following facts: In a

TABLE III.

Experiment 7. Effect of Intravenous Starch Suspension Injection on Respiratory Rate and Depth and on CO_2 Tension and pH of the Blood.

Time	Procedure	Respiratory rate per min.	Tidal air	Min. volume	Arterial blood					
					[CO_2]	pCO_2	pH	O_2 content	O_2 capacity	Per cent saturation
			cc.	*liters*	*mM*	*mm. Hg*		*mM*	*mM*	
12.12	Dog breathing room air	17	174	2.96	23.60	37.56	7.40	7.46	8.70	85.8
12.22	" "									
12.45–12.51	Injection of starch suspension									
12.53	Room air	46			25.10	40.82	7.39	7.26	8.96	81.1
1.00	" "	50	130	6.48	23.25	44.00	7.32	6.81	8.80	77.4
1.13	Dog breathing 95 per cent O_2									
1.20		50	123	6.18						
1.26					23.52	45.52	7.31	8.87	9.36	94.8

TABLE IV.

Experiment 9. Effect of Intravenous Starch Suspension Injection on Respiratory Rate and Depth and on CO_2 Tension and pH of the Blood.

Time	Procedure	Respiratory rate per min.	Tidal air	Min. volume	Arterial blood					
					[CO_2]	pCO_2	pH	O_2 content	O_2 capacity	Per cent saturation
			cc.	*liters*	*mM*	*mm. Hg*		*mM*	*mM*	
11.59	Breathing 95 per cent O_2 throughout experiment									
12.05		21	150	3.16	23.10	34.36	7.43			
12.17–12.26	Intravenous injection of starch suspension									
12.29		37	124	4.62	22.75	39.46	7.36	9.00	8.42	107

dog anesthetized with barbital-sodium, the intravenous injection of a suspension of potato starch grains resulted in an increase in respiratory rate from 17 to 50 per minute. This was associated with a decrease in tidal air amounting to 44 cc., or a 25 per cent decrease, and a resulting increase in the volume of pulmonary ventilation from 2.96 liters to 6.48 liters per minute. This change occurred while the animal was breathing room air and was accompanied by a drop in the oxygen saturation of the arterial blood from 85.8 per cent to 77.4 per cent. Accompanying this the pCO_2 rose from 37.56 mm. to 44.00 mm., with practically no change, however, in the CO_2 content of the serum as expressed in millimols. The hydrogen ion concentration, however, increased, as shown by the drop in pH from 7.40 to 7.32.

At this point oxygen was administered to the animal, with the result that the arterial oxygen saturation rose to 94.8 per cent, or the normal level, without, however, affecting the rate or depth of breathing, which still remained rapid and became even shallower than it had been. Nor was there a return of the pCO_2 or pH to the former level.

From these experiments it can be concluded that embolism due to intravenous starch suspension leads to rapid and shallow breathing, which is accompanied by a fall in the percentage oxygen saturation of the arterial blood, a rise in the pCO_2 and a fall in pH.

Restoring the blood to its normal oxygen saturation affects neither the respiratory rate or depth nor the carbon dioxide tension or hydrogen ion concentration. Similar results, as far as changes in pCO_2 and pH are concerned, were observed in Experiment 9, in which the animal was permitted to breathe oxygen throughout, so that the arterial blood remained completely saturated with oxygen. In this experiment the respiratory rate rose from 21 to 37 following starch injection, the tidal air falling from 150 cc. to 124 cc. A slight drop in the millimolecular concentration of CO_2 occurred, from 23.10 mM to 22.75 mM, but with a fall in pH from 7.43 to 7.36, the pCO_2 may be calculated to have risen by slightly more than 5 mm. These changes are brought out in Table IV.

Thus far no conclusions can be drawn as to whether the observed changes in pCO_2 and pH are the cause of the accelerated respirations or whether they result from the same condition which gives rise to rapid and shallow breathing.

To observe the effect on respiration of increasing the partial pressure of CO_2 in the serum without the disturbing influence of pathological changes in the lungs, such an increase was produced by permitting a dog to inhale from a Douglas bag containing a 10 per cent CO_2-90 per cent oxygen mixture. In this experiment (No. 12) in which the CO_2 tension of the serum was raised by CO_2 inhalation there was naturally an increase in the hydrogen ion concentration, both changes being consonant with those observed in the starch experiments. Instead of the development of rapid and shallow

TABLE V.

Experiment 12. Effect of Inhalation of 10 Per Cent CO_2-90 Per Cent O_2 Mixture on pCO_2, pH of the Blood and on Pulmonary Ventilation.

Time	Procedure	Respiratory rate per min.	Tidal air	Min. volume	$[CO_2]$	pCO_2	pH
			cc.	*liters*	*mM*	*mm.Hg*	
12.29	Breathing 95 per cent O_2	25.0	170	4.26			
12.35	" 95 " " "				26.21	40.76	7.41
12.38	" 95 " " "	30.4	194	4.90			
1.56	" 95 " " "	21.4	175	3.74			
2.03	" 95 " " "				26.28	42.82	7.39
2.05	" 95 " " "	21.4	180	3.86			
2.13–2.42	Breathing {10 per cent CO_2 / 90 " " O_2}						
2.30		42.0	390	16.42			
2.40					30.63	67.45	7.24
2.42		42.4	383	16.24			

breathing, however, the respirations, though they accelerated, more than doubled in depth. The average rate before CO_2 inhalation was 24, with a tidal air of 180 cc., compared to a rate of 42 after, with a tidal air of 383 cc. This was associated with a rise of CO_2 tension of nearly 25 mm., and a drop in pH from 7.40 to 7.24. These changes are exhibited in Table V.

This experiment (No. 12) is included simply for the purpose of demonstrating the well known effect of increasing the CO_2 tension and the hydrogen ion concentration, on the respirations. The significant difference from the starch effect is the marked increase in

depth. In many instances breathing high concentrations of CO_2 will increase the depth of respirations with little effect on rate.

Since, from the foregoing three experiments (Nos. 4, 7 and 9) it was impossible to say whether the changes in pCO_2 and pH antedated the onset of accelerated respirations, it was planned to draw the blood for analysis immediately after acceleration had begun. By this maneuver it was hoped to determine which change occurred first, *i.e.*, the change in CO_2 tension and pH or the change in rate and depth

TABLE VI.

Experiment 14. The Effect of Intravenous Starch Suspension Injection on Respiratory Rate and Depth, and on CO_2 Tension and pH of the Blood.

Time.	Procedure	Respiratory rate per min.	Tidal air	Min. volume	$[CO_2]$	pCO_2	pH
			cc.	*liters*	*mM*	*mm.Hg*	
11.43	Dog breathing 95 per cent O_2 throughout experiment	23.6	107	2.52			
11.49					24.40	51.48	7.27
11.52		23.2	120	2.78			
12.00–12.09	65 cc. 2 per cent starch suspension injected intravenously						
12.15		42.4	95	3.96			
12.21					23.58	49.75	7.27
12.24		47.6	89	4.24			
12.50					24.44	51.55	7.27
12.53		66.6	77	5.14			
1.09		73.0					

of respirations. It was found, in fact, that *accelerated respirations may occur before any measurable increase in CO_2 tension or hydrogen ion concentration*. From this the conclusion seems justified that the changes in CO_2 tension and pH are not the cause of rapid and shallow breathing which arises after embolism of the pulmonary arterioles and capillaries.

In Experiment 8 the respiratory rate, before starch injection, was 12 to the minute. At this time the pCO_2 was 66.95 mm. and the pH 7.23. After starch injection the rate had risen to 47 but the

pCO_2 was 63.42 mm. and the pH 7.25. Similarly with Experiment 14, in the control period the respiratory rate was 23 per minute and the average tidal air 113 cc. Starch injection resulted in a rate of 48 to the minute with a decrease in depth to 89 cc. No significant change, however, had occurred in the CO_2 tension or hydrogen ion concentration. The facts of this experiment (No. 14) are supplied in Table VI and in the appended protocol.

Protocol of Experiment 14. (*See Table VI.*)

Female, mongrel hound. Weight 12.2 kilos.

9.55. 3.36 gm. barbital-sodium, dissolved in 25 cc. distilled water, injected into the left leg vein.

10.05. Dog quiet, snoring. 10.30. Dog quiet, insensitive. Cannulated right jugular vein and right femoral artery.

11.00. Respirations 27. Pulse 205. Rectal temperature 38.4°C. Corneal reflex present.

11.08. Additional 5 cc. 5 per cent solution of barbital-sodium.

11.25. Respiratory mask adjusted to muzzle. 11.30. Breathing 95 per cent O_2 from Douglas bag. 11.41. Connected with Tissot spirometer.

11.43. First respiratory period. Expired air collected for 5 minutes. Total volume 12.6 liters. 118 respirations.

11.49. 25 cc. bright red arterial blood drawn from right femoral artery.

11.51. Second respiratory period. Expired air collected for 5 minutes. Total volume 13.9 liters. 116 respirations.

12.00. Injection of 2 per cent starch suspension begun. Respirations continuously counted.

12.01. Respiratory rate 26.

12.03. Total of 50 cc. starch suspension injected.

12.04. Respiratory rate 24.

12.09. Total of 65 cc. starch suspension injected. Respiratory rate 35.

12.14. Third respiratory period. Expired air collected for 5 minutes. Total volume 19.8 liters. 211 respirations.

12.21. 25 cc. bright red arterial blood drawn from right femoral artery.

12.23. Fourth respiratory period. Expired air collected for 5 minutes. Total volume 21.2 liters. 238 respirations.

12.46. Respiratory rate 61 per minute.

12.50. 25 cc. bright red arterial blood drawn from right femoral artery.

12.52. Fifth respiratory period. Expired air collected for 5 minutes. Total volume 25.7 liters. 333 respirations.

1.00. Rectal temperature 38°C.

1.01. Respiratory rate 72.

1.09. Respiratory rate 73.

At this time the experiment was brought to a conclusion and the animal used for another purpose.

SUMMARY AND CONCLUSIONS.

1. The production of multiple emboli of the pulmonary capillaries and arterioles results in rapid and shallow breathing which may be associated with anoxemia, but is not dependent for its occurrence upon anoxemia.

2. Similarly there may occur an increase in the partial pressure of CO_2 in the blood as well as an increase in hydrogen ion concentration.

3. These changes must be regarded as the result of the impaired pulmonary function.

4. They are not, however, the cause of the rapid and shallow respirations, since the abnormal type of breathing may occur without the attendant blood changes.

5. The characteristic type of response to increase in CO_2 tension is an increased rather than a decreased depth of respiration.

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THE EFFECT OF MULTIPLE EMBOLI OF THE CAPILLARIES AND ARTERIOLES OF ONE LUNG.

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Plate 23.

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INTRODUCTION.

The striking fact (Dunn (1), Binger, Brow and Branch (2)) that obstruction to the pulmonary arterioles and capillaries leads to a marked disturbance of the respiratory mechanism with the development of rapid and shallow respirations, and an increase in the minute volume of pulmonary ventilation, presents a pathological state which we have attempted to analyze further. Interest in this effect lies partly in its clinical application to such conditions as lobar pneumonia in which accelerated respirations are an important physical sign; and partly in the opportunity for investigating the difficult problem of respiratory rhythmicity and some of the factors influencing it. The reasons for thinking that an analogy exists between the rapid breathing due to multiple emboli of the arterioles and capillaries and the rapid breathing occurring in lobar pneumonia are several. In the first place in both conditions rapid breathing may occur independently of anoxemia. In the animal with multiple emboli this fact has been experimentally shown (2). And we have frequently observed patients with pneumonia continuing to breathe at the rate of 40 or more to the minute even after oxygen want has been relieved by oxygen administration. The occasional persistence of rapid respirations after crisis, when pulse and temperature have returned to normal, has suggested that the stimulus for accelerated respirations may be a local one resulting from the pulmonary lesion. A probable occurrence of capillary fibrinous thrombi has been described in pneumonic lungs by Kline and Winternitz (3), and that obstruction to the circulation may

occur in the consolidated lobe is known. This last fact again suggested a possible similarity in causes operating to produce similar effects.

Moreover, it has been found in experimental pneumonia produced in dogs by intratracheal insufflation of cultures of *B. friedlænderi* (Porter and Newburgh (4)) that the dyspnea which resulted could be checked by sectioning the vagi or blocking vagal impulses with cocaine. A similar slowing of accelerated respirations has been demonstrated for the tachypnea resulting from multiple experimental emboli. In this condition either vagal section (1) or vagal freezing (2) immediately brings about slow, deep breathing.

Quite aside from its immediate application to disease, a study of changes in respiratory rate has appeared important to us from the point of view of the nervous control of respiration. Of this subject not much is known. We know that the so called respiratory center sends out impulses which vary in strength and frequency, according to certain changes, physical and chemical, occurring either in the blood stream or in the center itself. And we know that the character of breathing, *i.e.* rate and depth, is influenced in some manner by impulses travelling along the vagus nerves (Le Gallois (5), Hering and Breuer (6), Head (7)). That these impulses are of a centripetal nature may be assumed from the studies of Einthoven (8) in which he demonstrated electrical changes in the thoracic end of the cut vagus occurring synchronously with changes in distension of the lungs. Whether there are centrifugal vagal impulses important to the control of respiration is not known. The vagus nerves in the dog, more than in the cat and rabbit, are not pure nerves, but are colonies containing sympathetic and depressor fibers as well. These nerves supply both heart and lungs. Cutting and freezing experiments are therefore necessarily varied in their effects and difficult to interpret.

The perhaps undue emphasis which we have placed on respiratory rate rather than on the minute volume of pulmonary ventilation lies in our interest in the nervous mechanism, where change in rate must represent a reversal in impulse occurring either peripherally in the breathing apparatus or in the respiratory center.

We must again emphasize the great difficulties introduced by the use of anesthetics. One is indeed caught between the horns of a

dilemma. To study the respiration in animals without an anesthetic is often impossible when concerned with changes involving the circulation in the lungs. We have previously (2) alluded to the effects of emotional and other adventitious stimuli which may confuse an experimental procedure. And yet equally confusing may be the sometimes excitatory, sometimes depressing influences of the anesthetic used to obviate the other effects. We have varied our technique, using luminal sodium by stomach tube, or barbital-sodium given intravenously or again ether with sterile technique and survival, in animals previously trained to lie still and breathe quietly. In the interpretation of results we have had constantly to keep in mind the effects of anesthetics.

The characteristic gross and microscopic pathology of the lungs of dogs in which multiple emboli of arterioles and capillaries have been produced by injection of suspensions of starch cells is congestion, edema and atelectasis. This is associated with a diminution in lung volume as determined by a measurement of the functional residual air (2). It seemed not improbable that these changes resulted in a decrease in elasticity of pulmonary tissue producing a shallow tidal air and thus, through the mechanism of the Hering-Breuer reflex, a rapid respiratory rate. Could the phenomenon be reproduced by causing similar lesions in one lung? If so, this would tend to establish more clearly the analogy with rapid and shallow breathing as it occurs in pneumonia. Experiments were planned with an eye to answering this question. It must be stated in advance that because of the technical difficulties involved and the necessary operative trauma the answers were not as unequivocal as was hoped.

EXPERIMENTAL.

Experiment 1. Effect of the Injection of Starch Suspension into One Lung.—A female dog, weighing 7 kilos, was given by slow intravenous injection 2.50 gm. barbital-sodium dissolved in distilled water, or 0.34 gm. of the drug per kilo of body weight. Within ½ hour the dog was quiet and relaxed, and breathing regularly at the rate of 13 respirations per minute. The animal was then intubated and artificial respiration started by the intratracheal insufflation of an interrupted current of air. With the dog lying on its right side the thorax was opened by incising the skin, muscles and pleura in the 4th left intercostal space. The ribs were separated by mechanical retractors, and with very little blunt dis-

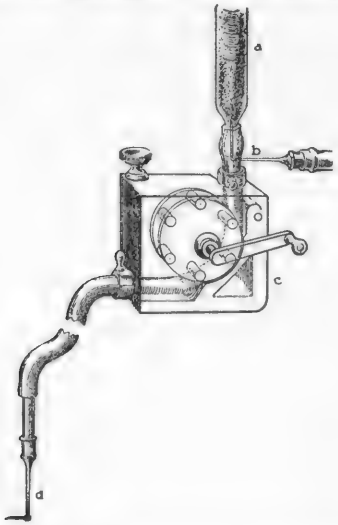
section the artery to the left lung was freed. By a method to be described below a suspension of starch grains (sufficient ordinarily to produce rapid and shallow breathing when injected intravenously) was injected directly into the artery supplying the left lung. Thereupon the animal's thorax was closed by approximating the 4th and 5th ribs by three stout ligatures, care being taken to distend the lungs and drive all air out of the pleural space before closing the chest. The muscle and skin layers were next repaired by suturing, the dog turned on its back and allowed to breathe spontaneously after removing the tracheal tube. To avoid the complications of anoxemia a stream of oxygen was blown through a funnel placed over the dog's muzzle. In this animal the preoperative respiratory rate varied from 16 to 20 breaths per minute. The postoperative rate, after the temporary acceleration had subsided, varied from 24 to 21. Following a steady respiratory rate at this level for 25 minutes the dog was killed by the intravenous injection of 20 cc. of a saturated solution of magnesium sulfate, and autopsied. The abdominal cavity and its viscera were normal. On opening the thorax the left lung, containing the starch grains, was mottled and paler than the right, which appeared normal. Frozen sections of the lungs, stained with Lugol's solution, showed the four lobes of the right lung to be free from starch grains, whereas in the small vessels of the left lung, especially in the upper and ventral lobes, they were thickly distributed.

From this experiment the following tentative conclusion can be drawn: Embolism of the capillaries and arterioles of one lung (the left) does not result in rapid and shallow breathing, when the other lung is functioning normally. The phenomenon is therefore not of an irritative nature due, solely, to the presence of the embolic material in one lung.

There is one important criticism to this experiment which obviously makes it inconclusive, namely: Was there actually enough embolic material introduced into the left lung to produce rapid and shallow breathing? It has repeatedly been observed that with intravenous starch suspension injections into the intact, unoperated animal a certain volume of suspension is necessary before accelerated respirations start. This fact was interpreted as indicating that the phenomenon was in some manner related to the degree of obstruction to the pulmonary circulation rather than the result of irritative, local stimuli set up in the lungs.

Method of Injecting Starch Suspensions Directly into the Artery Supplying One Lung.

As this method may prove to be of use to others it seems worth describing in detail. The method has the advantage of allowing the injection to proceed without clamping the artery or interfering with the circulation in the lung. The injection apparatus consists of a graduated burette to which about 10 inches of rubber tubing are attached. The tubing passes through a du Noüy* pump, which operates by rotating a wheel, designed to compress and decompress alternately



TEXT-FIG. 1. Apparatus for injecting suspensions into the pulmonary artery or its branches. *a*, burette; *b*, hollow needle through which a stream of air is blown to keep suspended particles from settling; *c*, du Noüy pump; *d*, bent needle with ball of solder in place.

the rubber tubing, thereby ejecting the fluid which enters the tubing from the burette. The speed and force of injection can be varied at will by varying the number of rotations of the wheel.

* This very useful pump, which was devised by Dr. du Noüy, of The Rockefeller Institute, has not previously been described in the literature. It was manufactured by the Central Scientific Company. We are indebted to Dr. du Noüy for its use.

The distal end of the rubber tube connects with a No. 21 gauge Luer needle 3 cm. long. 1 cm. from its point this needle has been bent at right angles by heating to a dull red, bending with a pair of nippers and then retempering. A small ball of solder is fused on to the needle at the right angle bend. Before thrusting the needle point through the arterial wall a small piece of muscle is "baited" on to the needle in much the same manner as baiting a fish hook. The ball of solder keeps the fragment of muscle from sliding around the bend and up the shank of the needle. This muscle fragment serves the double function of a flap valve over the puncture wound and of supplying tissue extracts which act as coagulants. With this equipment the arterial wall can be punctured with little hemorrhage and the needle kept in place for 15 to 20 minutes or more, without interfering with the flow of blood through the vessel. On removing the needle, the bleeding which usually occurs at the puncture wound can be controlled by the application of a fragment of muscle and cotton pledgets moistened in warm saline. Text-fig. 1 illustrates the burette, pump and needle used for injecting suspensions into the pulmonary artery or its branches.

Experiment 2.—An important corollary to the foregoing experiment, and one free from the criticism applicable to it, is the following, in which a dog, similarly anesthetized with barbital-sodium, was prepared for the experiment by ligating the left branch of the pulmonary artery. After the thorax had been closed, the animal was allowed to breathe oxygen. The respiratory rate at this time was 10 to the minute. A starch suspension of the same strength as in the previous experiment was then introduced into the circulation through a cannula in the right jugular vein. Since the left branch of the pulmonary artery had been ligated, all the starch entered the vessels of the right lung. The result was rapid and shallow breathing, the rate reaching a level of 58 to 60 per minute, where it remained for 1 hour before the animal was killed. The absence of anoxemia, as a contributing cause to the rapid breathing, was established by analysis of the dog's arterial blood at the close of the experiment, the percentage oxygen saturation being 100.

At this point, the tentative conclusion can be drawn that emboli in the capillaries and arterioles of one lung do not produce rapid and shallow breathing when the circulation through the other lung is normal. Or, in other words, the presence of embolic material, such as starch grains in one lung, does not excite this change in respirations. When, however, the circulation to the other lung is cut off by ligating its artery, emboli of capillaries and arterioles of one lung do produce rapid and shallow breathing in spite of the absence of oxygen want. Under the conditions of the experiment, the "embolized" lung adequately satisfied the animal's oxygen needs, but, even so, respirations assumed a definitely abnormal and accelerated character.

In an effort to surmount our own objections to the first experiment of this pair (Experiment 1), a new approach was devised.

A method was employed by which the left branch of the pulmonary artery could be occluded after the thorax had been closed and while the animal was breathing naturally. The occlusion was accomplished by inflating a pneumatic rubber cuff which had been previously placed about the left branch of the pulmonary artery. The method is given in detail in another paper in this *Journal* (9). Starch suspensions were then injected intravenously so that the emboli lodged in the vessels of the right lung. The left branch of the pulmonary artery was then released. By so doing, blood was allowed to circulate through the left lung after emboli, sufficient to produce rapid and shallow breathing, had lodged in the right lung. Under these conditions, does reestablishing the circulation through the left lung restore the respiratory rate to its original level? It should be stated that in these experiments too, anoxemia was prevented by oxygen inhalation.

The results of this series of experiments were variable. In some, reestablishing the circulation to the left lung (after production of rapid and shallow breathing by intravenous injection of starch granules which had lodged in the capillaries of the right lung) was accompanied by a definite slowing of respiratory rate. In others, the rate remained accelerated. In only one experiment (No. 3), was the respiratory rate restored to its slow, preoperative level.

In this experiment, the dog breathed at the rate of 16 per minute before it was operated upon, and the arterial blood was 97 per cent saturated with oxygen. The thorax was then opened and a pneumatic cuff placed about the left branch of the pulmonary artery. After closing the thorax, and allowing the animal to breathe naturally, the rubber cuff was inflated through a tube which projected through the chest wall. Inflation of the cuff completely shut off the flow of blood to the left lung. This resulted in no increase in respiratory rate. Indeed, at this stage of the experiment, the dog was breathing 13 times per minute. A starch suspension was thereupon injected intravenously, with the result that the respirations became shallow and accelerated to the rate of 61 per minute, despite the fact that the arterial blood was still 95 per cent saturated. The obstruction to the left branch of the pulmonary artery was next released by allowing the cuff to deflate, with the result that within 4 minutes the respiratory rate had dropped to 11 per minute.

This experiment is a clean-cut one and is more susceptible of interpretation than the others of this series. The dog breathed normally with only one lung, the right. When small emboli were introduced into the right lung, the respiratory rate accelerated to nearly 5 times

its previous rate. When, however, circulation was reestablished in the left lung, the respiratory rate returned to normal. Clearly, then, from this experiment too, one could conclude that emboli in the capillaries of one lung alone (the right) do not produce rapid and shallow breathing, unless the other lung is thrown out of the circulation, or, (as is suggested below), otherwise structurally damaged. That this last may account for the variable results referred to above is suggested by the following observations. In several experiments, the left lung, which had been temporarily deprived of its arterial blood, was, at autopsy, found to be bright red in color, instead of the normal salmon-pink. We know that depriving an organ of its blood supply may result in hyperemia from dilated capillaries, when the blood is again admitted (10). It is our opinion, though this fact is not definitely established, that in those animals in which accelerated respirations persisted after reestablishing the circulation to the left lung, some such capillary damage had been produced.

Experimental evidence for this statement is supplied by histological studies of the left lungs of Dogs 3 and 4. In Dog 3 (the experiment cited above in which the rate returned to normal on release of the cuff) the microscopic anatomy of the left lung is essentially normal. In Dog 4, however, in which the left lung, at autopsy, was noted to be bright red and granular in appearance, the respiratory rate remained at 61 per minute even after release of the obstruction to its artery. Here the microscopic picture is definitely abnormal, the thickness and distortion of capillaries being plainly visible. We reproduce photomicrographs of two typical regions of the left lungs of these two dogs which show clearly this difference in them (Figs. 1 and 2).

It should be stated that the variable behavior of different animals could not be correlated with variations in the percentage of oxygen saturation of the arterial blood, nor in the CO_2 tension or pH of the blood (11). Since this is true, we have not considered it worth while to present these data in this publication.

DISCUSSION.

When a suspension of potato starch grains is intravenously injected into a dog, the grains lodge in the finer arterioles and capillaries of the lungs. No change in the dog's respirations occurs until a certain, somewhat variable (in different animals) dose has been given. The

dog then develops rapid and shallow breathing, the respiratory rate often reaching 60 or more per minute. This phenomenon has interested us, partly because of an apparent analogy to the accelerated respirations seen clinically in cases of lobar pneumonia, partly because we believed that an understanding of this condition might throw some light on the problem of the rhythmicity of respiratory movements, and in particular upon the so called Hering-Breuer reflex.

Previous work has not revealed the cause of this abnormal type of breathing. It has not been correlated with (1) changes in arterial or venous blood pressures, (2) changes in percentage saturation of the arterial blood with oxygen, (3) changes in carbon dioxide tension or pH of the blood. It was found, however, to be associated with a reduction in lung volume, as expressed by measurement of the so called functional residual air, or that volume of air remaining in the lungs at the end of a quiet, normal expiration. And it was found that the characteristic pathological picture associated with this abnormal type of breathing was congestion, atelectasis and edema of the lungs.

The present study was undertaken to discover whether similar emboli introduced into one lung would result in a similar change in breathing. To accomplish this, that is, introduction of emboli into one lung, we had to resort, not only to the use of anesthetics, but to difficult and drastic operative procedures. Such procedures, of course, have drawbacks, and we have previously dwelt on the care necessary in interpreting results in the face of them.

Experimental evidence here presented appears to us to justify the conclusion that emboli lodged in the arterioles and capillaries of one lung produce an accelerated type of breathing only when the other lung has been excluded from the circulation by ligating or clamping its artery, or when the capillaries of the other lung have been abnormally distended by depriving them of blood for a period and then again admitting the blood.

The phenomenon would, therefore, appear to be in some manner related to the condition of diminution of the pulmonary vascular bed, to congestion in the lung or to resistance to the flow of blood through the lungs.

SUMMARY AND CONCLUSIONS.

1. Injection of a suspension of potato starch cells into the left branch of the pulmonary artery, in quantity sufficient ordinarily to give rise to markedly accelerated respirations, resulted in no change in respiratory rate.

2. A method for injecting substances into the pulmonary artery or its branches without interfering with the blood flow to the lungs has been described.

3. Injection of similar material into one lung when the other is excluded from the circulation either by ligation or by temporary clamping does give rise to rapid and shallow breathing (from a rate of 10 to 15 per minute to one of 60 or over) identical in character to that brought about by introducing emboli into both lungs.

4. A method for clamping and releasing the pulmonary artery or its branches in a dog breathing normally with closed thorax has been devised. This is described in detail in another paper.

5. After rapid breathing has been initiated by the effect of emboli lodged in the arterioles and capillaries of the right lung, reestablishing the circulation in the other lung by releasing the clamp on its artery may or may not restore the respiratory rate to its original, normal level.

6. This discrepancy in results has not been correlated with any difference in oxygen saturation of the arterial blood, or in carbon dioxide tension or pH of its plasma.

7. It is, however, believed to be related to the gross and microscopic anatomy of the lung of which the artery has been temporarily clamped. Photomicrographs are published, showing in one dog (No. 3), in which the respiratory rate returned to normal, a normal histological picture of the left lung, and in another dog (No. 4), in which the rate remained rapid after release of the clamp, a picture characterized by congestion and dilatation of arterioles and capillaries.

8. The fact that accelerated respirations result from emboli in the pulmonary capillaries and arterioles only after a certain quantity of material has been introduced, and the fact that emboli in one lung do not occasion accelerated respirations unless the circulation through the other lung is occluded or abnormal, leads us to the conclusion that

the phenomenon is not an irritative stimulus due to foreign bodies, but is in some manner related to (*a*) diminution of the pulmonary vascular bed, (*b*) resistance to the blood flow through the lungs or (*c*) congestion or dilatation of the arterioles and capillaries of the lungs.

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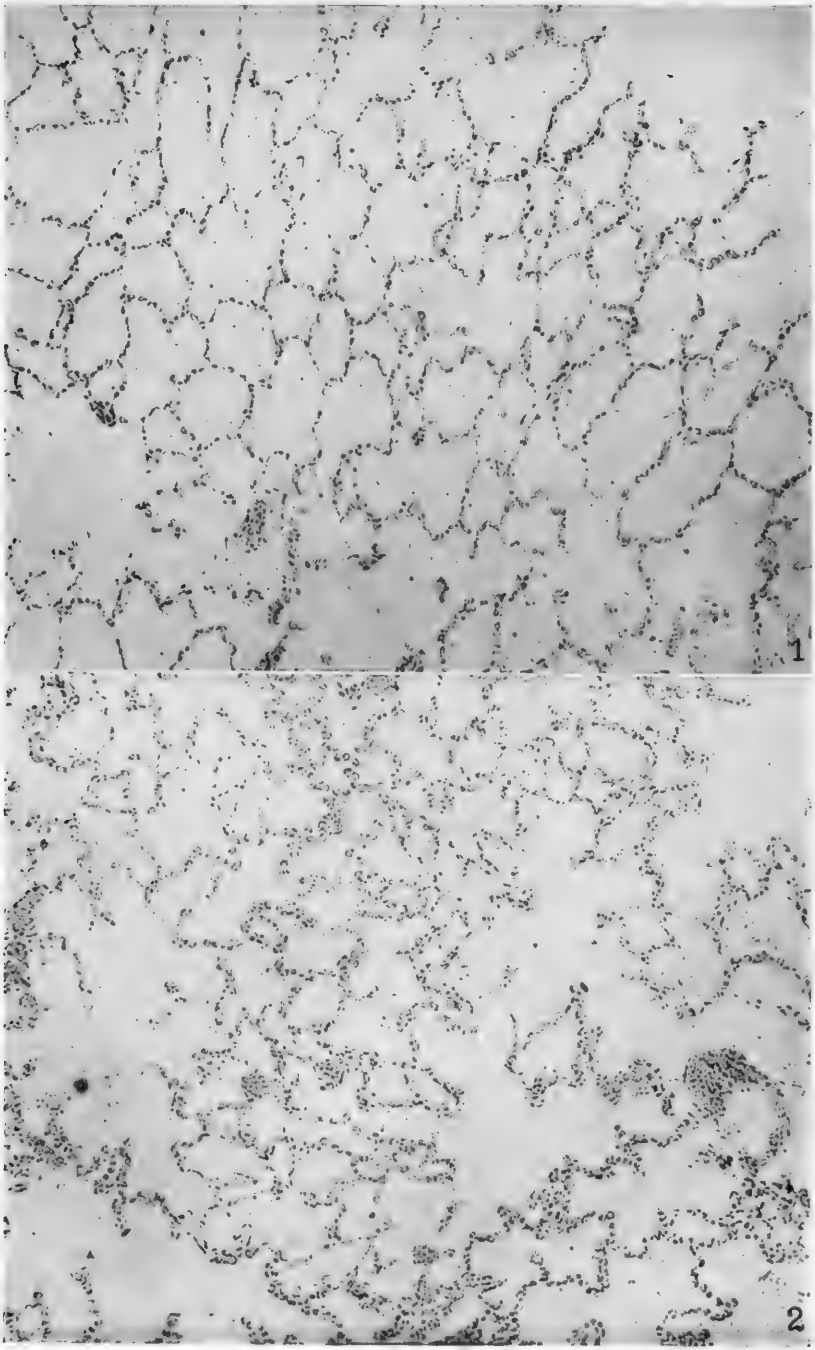
EXPLANATION OF PLATE 23.

FIGS. 1 and 2. Photomicrographs of sections of lungs from Experiments 3 and 4. × 130.

FIG. 1. Experiment 3. Section from left upper lobe. The histological picture is essentially normal.

FIG. 2. Experiment 4. Section from left upper lobe. The picture shows marked engorgement and tortuosity of alveolar capillaries.

Both preparations were made with similar technique, the dogs being killed by the intravenous injection of a saturated solution of magnesium sulfate. The lungs were fixed by immersion in Helly's fluid, after they had been distended *in situ* by the intratracheal injection of about 1 liter of Helly's fluid.



(Binger, Boyd, and Moore: Effect of multiple emboli in one lung.)

OBSERVATIONS ON RESISTANCE TO THE FLOW OF BLOOD TO AND FROM THE LUNGS.

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Plates 24 and 25.

(Received for publication, December 15, 1926.)

INTRODUCTION.

Previous studies (1–4) have led us to the view that the cause of rapid and shallow breathing which results from multiple emboli in the arterioles and capillaries of the lungs must be sought for in the secondary vascular changes following embolism. Neither the presence of the embolic material *per se* nor the chemical changes found in the blood can be regarded as the direct cause of the modified type of breathing. The phenomenon appears to be related in some manner to the diminution of the pulmonary vascular bed, to the resistance to the flow of blood through the lungs or to the state of congestion and edema of the lungs. It has already been shown (2) that obstruction to the larger branches of the pulmonary artery by the intravenous injection of seeds of various sizes results in a condition quite different from that due to capillary obstruction. The functional abnormalities in breathing thus produced were found to result wholly from the condition of oxygen want. That blocking the capillary bed should have a different effect from blocking some of the larger branches of the pulmonary artery is not hard to understand. The first might be described as an "effective blockade" in which the blood passing through the lesser circulation must necessarily meet resistance. In blocking some of the larger branches, however, the blood has an alternative route to follow, and such resistance may not be encountered.

The probability that resistance to flow of blood through the lungs would result in heightened pressure in the pulmonary artery and perhaps the right heart was borne out by the work of Haggart and Walker

(5) and of Wiggers (6). It occurred to us that such pressure changes might result in stretching of the walls of the pulmonary artery or of the right ventricle and that this might induce afferent stimuli which could occasion reflex changes in respirations. No experimental evidence for this assumption, however, can be adduced from this work, and the results here published, though perhaps negative from the point of view of explaining the origin of rapid and shallow breathing, are of themselves of interest, we believe, and have helped us with an understanding of the problem.

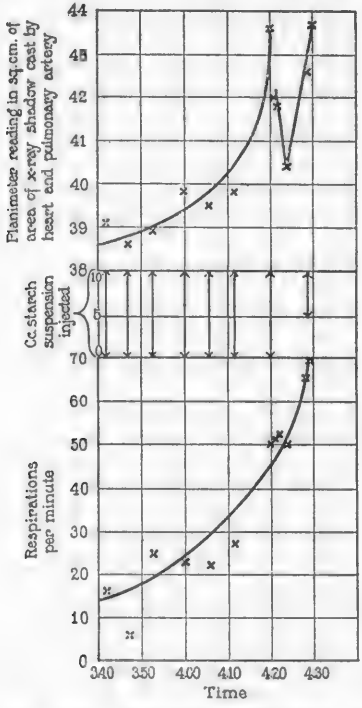
EXPERIMENTAL.

Dilatation of the Pulmonary Artery and Right Ventricle Following Obstruction to the Arterioles and Capillaries of the Lungs.

Without indulging in the artificial conditions necessarily prevailing in cardiac oncometry an effort was made to determine what changes occurred in the state of dilatation of the pulmonary artery and right ventricle after obstructing the flow of blood through the lungs by the intravenous injection of a suspension of potato starch grains. This was done by careful x-ray photographs made of the heart during injection of starch suspensions. A comparison of the photographs made under uniform conditions before and after the onset of rapid and shallow breathing shows a marked area of bulging at the base of the heart on the left side and a flaring out of the shadow cast by the right side of the heart. This was clearly shown in two experiments (Nos. 1 and 2). The x-ray photographs in Experiment 1 taken before and after the onset of rapid breathing following starch injection are reproduced in Fig. 1. The bulge at the left side of the base is plainly to be seen, as well as the flare in the shadow cast by the right side of the heart. At the time of the first picture the dog was breathing at the rate of 16 per minute; at the time of the second the respiratory rate was 64. The area of the cardiac shadow, measured by a planimeter, averaged 43.4 sq. cm. before the injection of starch as compared with 48.4 sq. cm. after. And the maximum transverse diameter of the cardiac shadow before embolism was 6 cm. as compared with 6.7 cm. after.

Like results were obtained in Experiment 2, in which x-ray photographs showed similar bulging at the left base of the heart and flaring

of the right border. By taking a series of x-ray plates during the experiment it was shown that these changes occurred synchronously with the acceleration of respirations. These facts are made plain by the two curves shown in Text-fig. 1 in which the lower represents



TEXT-FIG. 1. Lower curve: Ordinates, respirations per minute; abscissæ, time. Upper curve: Ordinates, area in sq. cm. as measured by a planimeter of x-ray shadow cast by the heart and pulmonary artery; abscissæ as in lower curve. The length of each arrow represents the volume of starch suspension injected at the time specified in the abscissæ.

respiratory rate per minute at the times specified in the abscissæ, and the upper, the area of the cardiac shadow in sq. cm. as measured by a planimeter. The arrows represent the times at which suspensions of starch were intravenously injected. The two curves will be

seen to vary synchronously and in the same direction. The break at the end of the upper curve may be regarded as a temporary recovery of muscular tone overcome by the injection of an additonal 5 cc. of starch suspension.

The planimeter measurements recorded here and in Experiment 1 were made by tracing the whole dense shadow cast by the heart, including the bulge at the left base which was suspected of being caused by the dilated pulmonary artery. By tracing the ventricular shadow alone from arbitrary points after the method of Levy (7) and Stewart (8), it was found that the increase in area was far less marked than it was when the bulge was included in the tracing. Direct inspection of the photographs shows the heart after embolism to be wider and shorter than before.

By carefully splitting the sternum without loss of blood in a dog* breathing at the rate of 56 per minute following starch embolism (Experiment 3) and maintaining the same respiratory rate by interrupted, intratracheal insufflation, after the chest had been opened, it was shown beyond doubt that the shadow of the bulge at the left base of the heart previously alluded to was cast by a greatly dilated pulmonary artery. This structure stood out a tense, bulging, sausage-like mass, three or four times its normal size.

The experimental facts thus far presented, we believe, prove that *obstruction to the capillaries and arterioles of the lungs results in an increase in the area of the x-ray shadow cast by the heart, and that this increase is the result partly of a widening of the transverse diameter of the ventricles, but chiefly of a marked bulging of the pulmonary artery. The changes in the heart and pulmonary artery were shown to occur synchronously with the acceleration of respiratory rate.*

The hypothesis on which we proceeded, and which was found to be faulty, was that the dilated pulmonary artery sent out impulses which reflexly accelerated respirations. Though the results of these experiments are in a sense negative, by eliminating false causes, they largely clarify our problem.

Experiments were devised with an eye to obstructing the blood flow to the lungs proximal to the pulmonary parenchyma itself so that

* The animal was anesthetized with barbital-sodium.

any changes in breathing could be attributed wholly to the cardiovascular effect, unclouded by pathological changes in the lungs. Two methods were used to accomplish this end: (1) the exposed heart method of Drinker; (2) a new method which will be described in detail below.

Experiments with the Drinker Exposed Heart Preparation.

By the technique devised by Drinker (9) the heart of a cat was exposed through a window cut into the sternum and the thorax closed by stitching the split pericardium to the edges of the window. The heart thus everted and supported on its pericardial sling, could be observed and its great vessels manipulated at will while the animal breathed without the aid of artificial respiration. To obstruct the flow of blood through the pulmonary artery, or one of its branches, a clamp and ligature were placed about it, after the method described by Haggart and Walker (5), in such a manner that progressive tightening of the ligature resulted in progressive diminution of the calibre of the vessel until it was completely occluded.

This experiment was performed on two cats anesthetized with barbital-sodium. In one of them (No. 4) the clamp was placed on the left branch of the pulmonary artery, in the other (No. 5) on the main trunk of the artery. Since the results of these two experiments are in complete agreement with those of Haggart and Walker (5) they will not be given in detail. In Experiment 4, in which the left branch of the pulmonary artery was suddenly occluded, no change occurred in the animal's respirations. In Experiment 5, in which the main trunk of the pulmonary artery was gradually compressed, no change in respiratory rate or depth occurred until a point was reached when respirations ceased entirely.

Because all of our previous studies on the effect of pulmonary embolism on breathing had been made on dogs, it was determined to repeat the last two experiments on dogs instead of cats. This, however, brought us face to face with a real difficulty. The Drinker exposed heart preparation, though admirable in the cat, cannot be used satisfactorily in dogs because of the depth of the dog's thoracic cavity, and the difficulty to effect a tight closure between the pericardial edges and the chest wall. Moreover, it seemed desirable to devise a

method in which the thoracic cavity was not restricted in size and one which could be used, if need be, in experiments made without anesthesia, on animals previously prepared by sterile, survival operations.

The method finally achieved was the following: A thin walled, flat rubber bag measuring 1.8 × 5.5 cm. communicating with a 30 cm. length of rather heavy walled rubber tubing (3 mm. in cross-section with a 2 mm. bore) was passed under the previously exposed and freed pulmonary artery.* By surrounding this rubber bag or cuff with a stocking of closely woven silk, to the four corners of which ligatures were attached, the rubber bag could be fastened in position about the vessel. The silk covering prevented overdistension of the bag on inflating it, and assured a uniform compression on the vessel, similar to that produced by the ordinary blood pressure cuff. Inflation of the bag was made through the patent end of the rubber tubing. The pressure in the cuff could be accurately controlled by the use of a mercury manometer. Fig. 2 illustrates the bag in place before the ligatures were fastened. When this is done, the bag forms a closely fitting cuff surrounding the vessel wall. Inflation immediately compresses the vessel. It was found that a pressure of 150 mm. Hg was more than sufficient to obliterate the lumen of the artery. As has been said, the method could be used in survival experiments in which the pulmonary artery or one of its branches could be occluded without the use of anesthetics, and after the effect of operative trauma had subsided. Fig. 3 is a photograph of such a survival experiment (No. 6) to be referred to below. The rubber tube, projecting through the chest wall, through which the bag is inflated, is shown in the picture.

Effect on Respiration of Compression of the Left Branch of the Pulmonary Artery in the Anesthetized Dog.—Dogs anesthetized by the intravenous injection of barbital-sodium were operated on, with the aid of artificial respiration, an incision into the pleural cavity being made through the 4th left interspace. Spreading the 4th and 5th ribs gave free access to the left branch of the pulmonary artery after the upper and middle lobes of the left lung had been carefully retracted downward and packed out of the field. The left branch of the pulmonary artery was surrounded by the pneumatic cuff described above and the chest wall closed by approximating the spread ribs with three stout ligatures after proper distention of the lungs. In closing the thorax, the free end of the rubber tube which communicates with the cuff was permitted to project through the chest wall.

* When one of the two main branches of the pulmonary artery was compressed, a somewhat smaller bag was used, *viz.*, 0.8 × 4.2 cm.

The effect on breathing of compressing the left branch of the pulmonary artery was noted in a series of experiments. The results obtained are similar to the cat experiment (No. 4) cited above. Blocking the circulation to the left lung produces essentially no immediate (2 minutes to 2 hours) effect on respiratory rate. Five animals showed a slight drop in rate, −1, −3, −3, −1 and −2, respectively. One animal showed no change. Eight animals showed a slight increase in rate, +2, +6, +1, +5, +1, +3 and +1. Whereas the average post-operative control rate was 22.5 per minute, the average rate after compression of the pulmonary artery was 24.2 The periods of compression, during which the respiratory rates were observed, varied from 2 minutes to 2 hours. Anoxemia was prevented from arising in these experiments by allowing the animals to inhale oxygen.

From these observations it can be definitely concluded that *restriction of the pulmonary vascular bed by nearly half does not result in rapid and shallow breathing.*

In two of this series of experiments (Nos. 7 and 8), the carbon dioxide tension and hydrogen ion concentration of the serum were studied before and after excluding the left lung from the circulation.

In Experiment 7 there was an increase of nearly 10 mm. Hg in pCO_2 with a corresponding drop in pH from 7.34 to 7.27. In Experiment 8, however, the pCO_2 fell from 54.05 mm. Hg, before clamping the left branch of the pulmonary artery, to 50.35 mm. Hg after, and the pH rose from 7.29 to 7.32. Tables I and II give the analytical data of these two experiments.

No constant effect, therefore, on the CO_2 tension or pH of the serum is to be anticipated from suddenly shutting off the circulation to the left lung.

Effect on Respiration of Gradual Compression of the Main Trunk of the Pulmonary Artery in the Surviving Unanesthetized Dog. Protocol of Dog 6.—(See Fig. 3.) February 3, 1926, a white and black, male setter, weighing 14.5 kilos, was given ether by cone after a preliminary injection of morphine. When the dog was fully relaxed a rubber tube was inserted into the trachea. Artificial respiration and anesthesia were maintained by forcing an air-ether mixture intermittently into the lungs at a pressure of 20 mm. Hg. The dog was placed on its right side, with fore legs extended. Thorax was shaved and skin scrubbed with soap, water and alcohol. The chest was opened by resecting the 5th rib from its costochondral junction laterally for a distance of about 3 inches. By a self-

retaining mechanical retractor the edges of the wound were held apart and the left lung was retracted from the field by a cotton pad moistened with warm, physiological saline solution. A vertical incision was then made in the anterior surface of the pericardium directly over the pulmonary artery, from the point at which

TABLE I.

Experiment 7.

Time	Procedure	Respirations	$[CO_2]$	pCO_2	pH
		per min.	*mM*	*mm.*	
1.41	Postoperative control period. Cuff in place but deflated. Dog breathing oxygen throughout experiment	20	27.56	50.40	7.34
1.46	Cuff inflated to 150 mm. Hg				
2.10		26			
2.40		24			
3.25		26	28.45	59.60	7.27

TABLE II.

Experiment 8.

Time	Procedure	Respirations	$[CO_2]$	pCO_2	pH	O_2 capacity	O_2 content	O_2 saturation
		per min.	*mM*	*mm.*		*mM*	*mM*	*per cent*
2.14	Postoperative control period. Cuff in place but deflated. Dog breathing oxygen throughout experiment	24	26.76	54.05	7.29	8.97	8.88	99
2.17	Cuff on left branch of pulmonary artery inflated to 150 mm. Hg							
2.52		25	26.71	50.35	7.32	9.02	9.10	101
2.52	Cuff deflated							
3.24		25	26.63	49.35	7.33	9.17	9.06	98.8

the pericardium is reflected from the great vessels downward for a distance of 2 inches.

The edges of the pericardium were retracted by temporary ligatures. The pulmonary artery was freed from the aorta by blunt dissection and the cuff tied snugly in place. After approximating the edges of the pericardium, the wound

was closed in layers, the tube connecting with the cuff being led through the incision as far as the platysma muscle, then beneath this muscle, to make its exit through the skin at a point on the dorsolateral aspect of the chest, 3 inches distant from the wound. This was an added precaution taken against the possibility of infection working its way in from the outside. Silk ligatures and sutures were used throughout.

The dog made a splendid recovery and on February 9, 1926, appeared well and acted normally. The only sign of infection was a slight, odorless discharge, which could be expressed about the tube at the point of its exit in the skin. Weight on this date, 13 kilos. The dog had shown a very decided disinclination toward food, but there had been some improvement in this respect over the immediate postoperative period.

February 9, 1926. On four separate occasions the tension in the cuff was gradually raised to the point of producing respiratory failure, without any increase in respiratory rate. Each break was characterized by identically the same symptoms. The dog would suddenly become restless, lift up its head and then begin to whine. This was immediately followed by muscle rigidity, and the respirations, which had become irregular, abruptly ceased. Immediate deflation of the cuff restored the animal to its normal state within a few seconds. The results obtained during the first and second compressions on this date are shown in Tables III and IV.

Similar results were obtained in another dog (No. 10) of this series. In this experiment it was observed at operation that inflation of the rubber cuff resulted in a marked dilatation of that portion of the pulmonary artery proximal to the cuff.

The conclusion can now be definitely drawn that *resistance to the flow of blood to the lungs obtained by sudden or gradual compression of the pulmonary artery, in both the cat and the dog, though it results in dilatation of the pulmonary artery and of the right chambers of the heart, does not give rise to rapid and shallow breathing as do multiple emboli of the pulmonary capillaries and arterioles, but produces practically no change in respirations until syncope and respiratory failure suddenly occur.* From this it is likewise concluded that the markedly accelerated and shallow respirations following embolism of the pulmonary capillaries and arterioles are not the result of a reflex stimulus arising in the dilated heart or pulmonary artery.

Effect of Gradual Compression of the Pulmonary Veins on the Heart and Respirations.—It remained to discover what effect impeding the return of blood from the lungs to the heart would have on the respira-

TABLE III.

Dog 6.

February 9, 1926. Sudden Compression of Pulmonary Artery in an Unanesthetized Dog.

Time	Tension in cuff	Respiratory rate	Pulse rate	Remarks
a.m.	*mm. Hg*	*per min.*	*per min.*	
11.30	0	19	135	Dog quiet
11.35	0	19	168	
11.36	0	16	124	
11.39	0	18		
11.40	100±	0		Cuff abruptly inflated. Respirations ceased. Cuff released
11.41	0	20		
11.43	0	20	124	

TABLE IV.

Dog 6.

February 9, 1926. Gradual Compression of Pulmonary Artery in an Unanesthetized Dog.

Time	Tension in cuff	Respiratory rate	Pulse rate	Remarks
p.m.	*mm. Hg*	*per min.*	*per min.*	
3.22	0	20		Dog quiet
3.23	0	22		
3.24	0			Inflation begun
3.25	40	24		
3.30	36	20	152	
3.32	50	21	147	
3.35	47	24		
3.38	62	24		
3.43	74	23		
3.45		24	145	
3.49	85	24	176	
3.52	106	26–0		Respirations ceased
3.58	0	20		

tory rhythm. Multiple embolism of the pulmonary capillaries and arterioles, we know, results in a condition of congestion and edema of

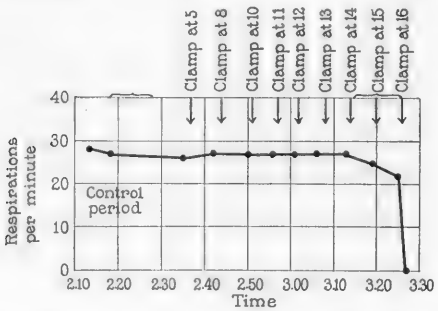
the lungs. That this change in structure might be responsible for the altered function was long suspected. Drinker, Peabody and Blumgart (10) showed that compression of the pulmonary veins in the cat resulted in a diminution in lung volume as measured by the volume of air which could enter the lungs under a uniform pressure. This diminution was attributed to the state of engorgement induced in the pulmonary capillaries. When the resistance to the outflow of blood was sufficiently severe and prolonged a condition of pulmonary edema arose. Their work included no functional studies on respiration, as

TEXT-FIG. 2. Graphic spirometer tracing made by cat in Experiment 11. The first portion of tracing was taken during the control period. The second portion of the tracing was taken toward the latter part of the experiment in which the pulmonary veins were being progressively compressed by a ligature. At *a* and *b* the ligature was still further tightened. The intervening respiratory rates may be seen in Text-fig. 3.

the animals (cats) were curarized and, therefore, not breathing spontaneously.

We again availed ourselves of the Drinker exposed heart preparation, and in a series of experiments on cats, we gradually compressed the pulmonary veins and observed the effect on the heart and on the respirations. To our surprise exactly the same "all or none" relationship obtained as in compression of the pulmonary artery. Gradually increased pressure exerted by a clamp and ligature on the pulmonary veins leads, after a certain point, to acute dilatation of the heart, followed by cessation of respiration. In no experiment, even when pressure was exerted for many minutes at the point just before respira-

tory failure occurs, did respirations become rapid and shallow. In Text-fig. 2 a graphic tracing is shown of the respirations and heart beat during gradual compression of the pulmonary veins in a cat. The first part of the record is the control period with the ligature in place but slack. It corresponds to the first bracketed zone in Text-fig. 3. The second portion of the record represents heart rate and respirations toward the end of compression corresponding in time to the second bracketed zone in Text-fig. 3. The intervening portion of the graphic spirometer tracing is omitted, but the rate may be seen in Text-fig. 3. During this whole second period the heart was blue and markedly dilated. Respirations will be seen to continue at about uniform rate



TEXT-FIG. 3. The respiratory rate of the cat in Experiment 11. The bracketed areas in the curve correspond in time to the two sections of the graphic tracing shown here (Text-fig. 2).

and depth until they more or less suddenly cease. This observation was repeated in two other cats in which it was shown that release of the ligature was promptly followed by return of the heart to its normal appearance and the respirations to the control rate and depth. *In no experiments did rapid and shallow breathing develop as the result of gradual compression of the pulmonary veins or, in other words, of increased resistance to the flow of blood from the lungs.* It was, unfortunately, impossible to compress the pulmonary veins in the dog because, as has been said, this animal is anatomically unsuited to the Drinker exposed heart technique; nor is the pneumatic cuff method applicable, so obscurely and closely packed are the veins as they enter the posterior auricular wall.

DISCUSSION.

Since this paper constitutes the last of this series of experimental studies on respiration it may be well, even at the cost of redundancy, to consider again our findings, and what lessons are to be gleaned from them. The work was originally undertaken with a hope of understanding more clearly the mechanism and significance of that type of breathing frequently encountered in patients suffering from lobar pneumonia,—breathing characterized by being both rapid and shallow. Such disordered respiration is known to be accompanied often by oxygen want, and the point has been raised that it may indeed be responsible for oxygen want which, in turn, through its influence on the respiratory center, may perpetuate the disordered type of breathing and lead eventually to the exhaustion and ultimate collapse of the respiratory organ system.

Our work began some years ago with a quantitative study of the degree of pulmonary involvement which occurred in lobar pneumonia (11). By measuring the so called "functional residual air," or that volume of air enclosed in the lungs at the end of a normal, quiet expiration, we arrived at a more or less quantitative estimation of the degree of pulmonary involvement. It was found in general that this function varied with the clinical course of the disease, that after crisis the volume of the functional residual air rapidly increased, and that it remained either stationary or decreased as long as the active infection persisted. No unequivocal correlation, however, could be arrived at between lung volume, so measured, and the type of breathing. Occasionally rapid and shallow respirations were seen to persist for a considerable length of time after the disease process had become arrested. Nor did the state of anoxemia seem to be responsible for the accelerated respirations. In most patients in whom oxygen inhalation relieves the existing state of anoxemia, as determined by oxygen analysis of the arterial blood, rapid respirations still persist.

There seemed to be a hitherto unconsidered element responsible for the disordered breathing and for various reasons this was assumed to be one of a nervous reflex nature. The work of Porter and Newburgh (12) showed that the dyspnea associated with experimental lobar pneumonia in dogs could be checked by sectioning the vagus

nerves or blocking them with cocaine. Dunn (13) discovered the interesting fact that multiple embolism of the pulmonary arterioles and capillaries in goats resulted in very rapid and shallow breathing which could at once be stopped by double vagotomy. Our studies began where Dunn's left off. We found that this phenomenon was inherently related to obstruction of the capillaries and arterioles. When the larger branches of the pulmonary artery were obstructed a similar disorder of breathing resulted, but this was found to be wholly the result of oxygen want and could accordingly be arrested or prevented by oxygen inhalation. Anoxemia of this origin was demonstrated to be due to a relative change in rate of blood flow through the pulmonary vessels. Obstruction of the finer vessels, though often associated with anoxemia, was not in this respect the responsible factor for the disordered breathing, since rapid and shallow breathing persisted even after relief of oxygen want.

What then was the cause of rapid and shallow breathing due to capillary embolism? To this problem we addressed ourselves and it was found far more difficult in its solution than had been anticipated. Indeed a proper understanding of it has depended upon the exhibition of a large amount of so called negative evidence.

We know that capillary obstruction is associated with a diminution in the volume of the "functional residual air" which is probably dependent upon the secondary state of edema, atelectasis and congestion which arises. It seemed reasonable to believe that this pathological condition of the lung, which could give rise to oxygen want, might likewise be responsible for a retention of carbon dioxide, or an increase in the pressure of CO_2 and the concentration of hydrogen ions in the blood. These changes were, in fact, found but that they are not the cause of the disordered breathing is strongly indicated by the fact that rapid and shallow breathing may occur after embolism without any increase in pCO_2 or fall in pH. We know, too, that the actual presence of the embolic material is not responsible for the accelerated breathing in the sense of its being a local irritant, though the inhibitory effect of vagotomy or vagal freezing first made us suspect this to be true. The fact that starch emboli do not produce rapid and shallow breathing solely by acting as local irritants is shown by these two considerations: (1) a considerable amount of embolic mate-

rial may be present without any influence upon the normal respiratory rhythm; (2) emboli may be present in the capillaries and arterioles of one lung sufficient ordinarily to produce rapid breathing without however, any alteration in normal breathing unless the circulation to the other lung is cut off or has been disturbed by a previous period of obstruction. These last facts suggested that the phenomenon was in some manner related to (1) a restriction of the pulmonary vascular bed, (2) resistance to the flow of blood through the lungs or (3) the state of congestion and edema of the lungs. The first two of these possibilities might easily be related to an extra burden on the heart, resulting in an increased pressure in the pulmonary artery and right side of the heart with a subsequent dilatation of these tissues. The demonstrastration by Levy (7) of an increased heart size in pneumonia was thought perhaps to be a germane phenomenon. It has been shown in this paper that dilatation of the pulmonary artery and right heart does in fact occur when the arterioles and capillaries of the lungs are obstructed. And it was tentatively hypothecated that the dilatation of the pulmonary artery and right heart resulting from obstruction to the finer vessels of the lungs occasioned impulses which reflexly accelerated respirations. Evidence has been brought forth in this paper to show that this hypothesis is erroneous and that reduction of the pulmonary vascular bed, at least by half, produces no change in breathing. Moreover, both resistance to the flow of blood to and from the lungs though followed, when of sufficient grade, by dilatation of the pulmonary artery and right heart, does not give rise to rapid and shallow breathing. The result of such resistance is an "all or none" phenomenon in which respirations continue at their normal rate and depth until they more or less suddenly cease completely.

We are left then to a consideration of the third possibility enumerated above, namely, that rapid and shallow breathing of the kind here described is the result of the particular lesion produced in the lungs, namely, congestion and edema.

How this acts to produce accelerated breathing still remains more or less a mystery: whether directly through irritation of vagal nerve ending; whether as the result of marked encroachment on lung volume; or whether through changes in elasticity of the lung, which to be sure we have not been able to demonstrate in the embolized lung at autopsy.

The first of these three possibilities seems to us at present to be the most likely. It is tempting to theorize but perhaps unwise to befog an already complicated subject by unproven hypotheses. Certainly congestion of the lungs, reduction in lung volume and impaired elasticity of the pulmonary parenchyma are all three intimately associated phenomena, and are present in many of the clinical states in which disordered breathing is a prominent symptom.

SUMMARY AND CONCLUSIONS.

1. Embolism of pulmonary arterioles and capillaries produced by the intravenous injection of starch grains results in a dilatation of the pulmonary artery and the right chambers of the heart. This has been demonstrated both by x-ray studies and direct inspection.

2. The dilatation of the pulmonary artery and heart occurs synchronously with the acceleration of respirations.

3. Dilatation of these structures produced by other means, such as obstruction to the flow of blood to and from the lungs, by gradually clamping either the pulmonary artery (cat and dog) or pulmonary veins (cat) does not, however, give rise to rapid and shallow breathing.

4. The effect of these maneuvers on respiration does not become apparent until respirations suddenly cease.

5. Neither does sudden restriction of the pulmonary vascular bed by clamping the left branch of the pulmonary artery give rise to rapid and shallow breathing, though this procedure may cause an increase in CO_2 tension and in hydrogen ion concentration of the blood.

6. Since rapid and shallow breathing is *not* the result of (1) anoxemia, (2) increased pCO_2 and hydrogen ion concentration of the serum, (3) restriction of pulmonary vascular bed by nearly half, (4) increase in resistance to the flow of blood to and from the lungs, (5) the presence of starch grains in the lungs acting as a local irritant, it must be the result of the secondary pathological changes which occur in the pulmonary parenchyma following embolism.

7. The nature of these changes, congestion and edema, has been discussed elsewhere. Whether they operate directly on nerve endings or through their influence on lung volume and tissue elasticity is not certain.

8. Various important clinical analogies have been emphasized.

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EXPLANATION OF PLATES.

PLATE 24.

FIG. 1, *a* and *b*. X-ray photograph of dog's heart taken in Experiment 1. Tube distance 2 meters. In *a* the dog was breathing 16 times to the minute, the area of the shadow as measured by a planimeter being 43.4 sq. cm. In *b* after a starch suspension had been injected intravenously, respirations were 64 to the minute, and the area of the shadow is 48.4 sq. cm.

PLATE 25.

FIG. 2. Operative field with pulmonary artery exposed and cuff in place ready for ligation.

FIG. 3. Dog 6, 8 days after operation, showing tube, which communicates with cuff surrounding the pulmonary artery, projecting through the chest wall.

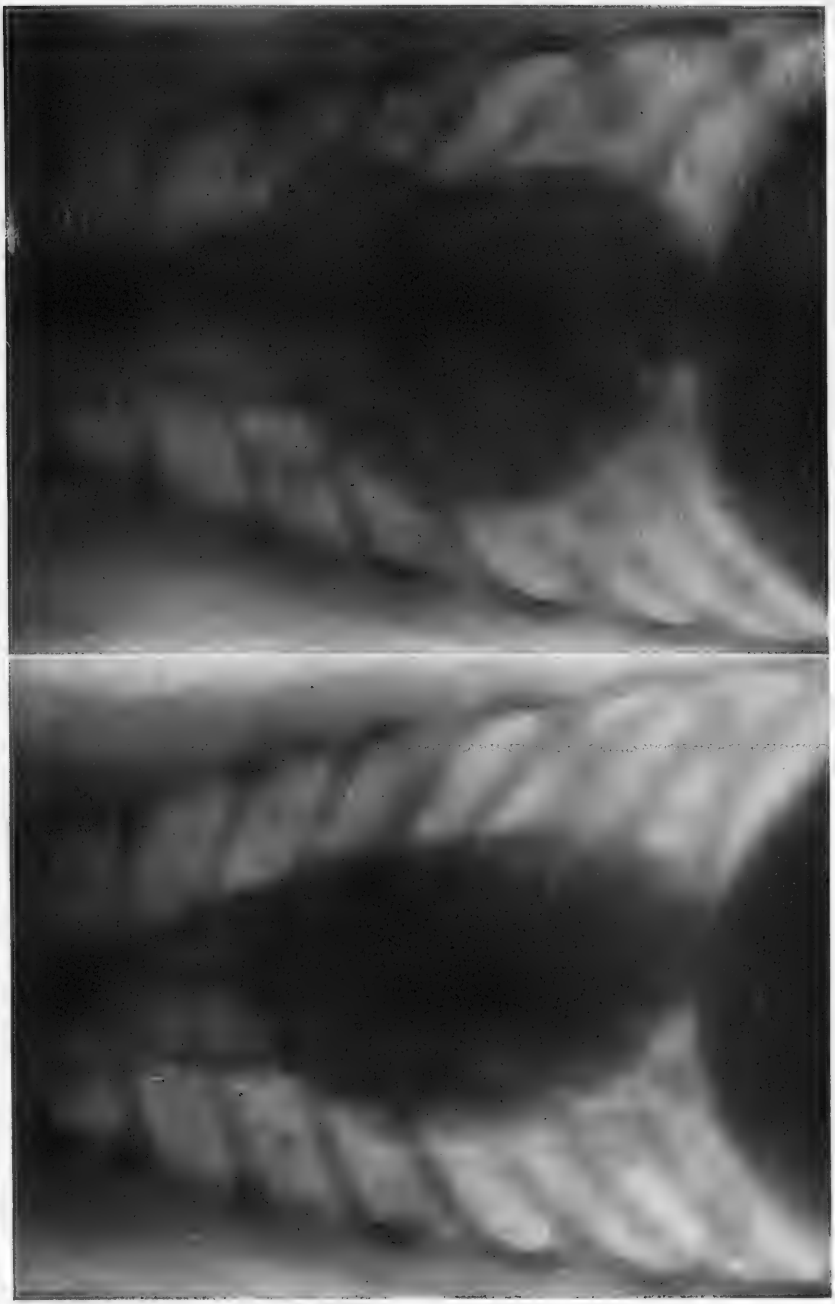

FIG. 1, *b*.

FIG. 1, *a*.

(Moore and Binger: Blood flow to and from lungs.)

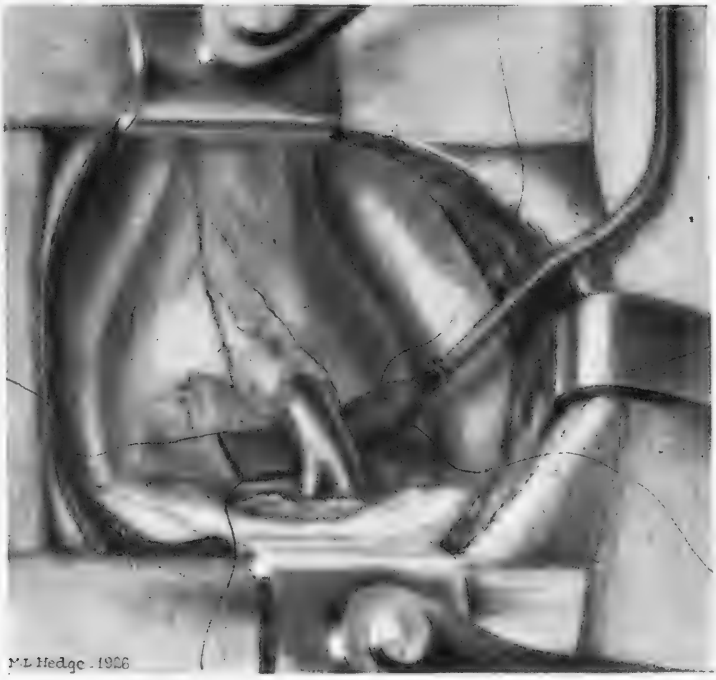

FIG. 2.

FIG. 3.

(Moore and Binger: Blood flow to and from lungs.)

STUDIES ON THE PHYSICAL AND CHEMICAL PROPERTIES OF THE VIRUS OF FOOT-AND-MOUTH DISEASE.*

I. Description of the Strain of Virus Used. Titration and Centrifugation Experiments.

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(Received for publication, December 16, 1926.)

The findings of the Commission to Study Foot-and-Mouth Disease will be presented in detail in a forthcoming publication by the United States Bureau of Animal Industry. A preliminary paper has already appeared[1] in which were reported comparative studies on vesicular stomatitis and foot-and-mouth disease. In a series of papers to be published in this *Journal*, of which this is the first, the writers will describe their experiments on the physical and chemical properties of the foot-and-mouth disease virus, and their attempts at its cultivation. Of the Commission's personnel, the writers were mainly interested in these particular problems. There was, however, a free interchange of ideas and suggestions, and a hearty cooperation among all the colleagues, so that all publications of the Commission may be regarded as the result of collaboration as a unit.

* The Commission to Study Foot-and-Mouth Disease was formed by the United States Department of Agriculture, under the direction of Dr. John R. Mohler, Chief of the Bureau of Animal Industry. It comprised Dr. Harry W. Schoening, of the Bureau of Animal Industry, Dr. Jacob Traum, of the Department of Veterinary Science, University of California, and, as Chairman, Dr. Peter K. Olitsky, of The Rockefeller Institute for Medical Research. Dr. Louis Boëz, of the Institute of Hygiene, joined the Commission later as a collaborator.

The writers wish to express their gratitude to the Doyen, Professor Weiss, to Professors Borrel, Vlès, and others, of the Institute and the University of Strasbourg, for their active and whole hearted cooperation.

[1] Olitsky, P. K., Traum, J., and Schoening, H. W., *J. Am. Vet. Med. Assn.*, 1926, lxx (N. S. xxiii), 147.

In practically all of the experiments to be reported in the series of papers, we employed, except in a few instances, one strain of foot-and-mouth disease virus. This strain had the advantages of causing lesions promptly and regularly, of acting powerfully, of inducing in practically all instances well marked secondary lesions, and of bringing about relatively few deaths in the experimental animals, thus permitting a long period of observation. The animal used in these studies, unless otherwise stated, was the guinea pig.

Description of Strain of Virus.

On June 18, 1925, through the kindness of a local veterinarian, Dr. Fuchs, a herd of cattle with typical foot-and-mouth disease was placed at our disposal for study. The cattle were kept on a farm about 15 kilometers from Strasbourg. Of twelve animals in the same barn, all in different stages of the disease, one cow was selected which was the last to be infected, and in the 3rd day of illness. She drooled considerably, and had broken aphthæ about the lips, tongue, and buccal mucosa, but no obvious foot lesions, and her temperature was 41°C. It was difficult to aspirate the exudate in the vesicles so that in the end what was brought to the laboratory was an admixture of this cow's saliva and stable refuse. This material was diluted 1:3 with Tyrode solution and then injected, unfiltered, intradermally in the hairless skin of the posterior pads of five guinea pigs. Typical primary vesicles appeared at the sites of injection in 24 to 48 hours in all five. From that time until the investigations were terminated, about a year later, the virus (Strasbourg or "Str." strain) was propagated in the guinea pig for 261 consecutive passages in a pure state, except on two occasions in late passages, when staphylococci were found in the vesicular exudate. These latter were then removed by filtration, without harming the virus. It was found later, by failure of cross-immunity with Vallée's A type, that the Str. strain was probably identical with that of the Vallée "O."

It is to be noted from the effects of transferring the impure virus from the cow to the guinea pig that the virus, when admixed with contaminating microorganisms, could free itself of ordinary bacteria in the tissues of a susceptible animal. Thus a property common to

many filter-passing viruses is shown by the active agent of foot-and-mouth disease. Indeed, after the first series of transfers, no extraordinary care was taken for the cleanliness of the site injected; that this was a proper mode of procedure is gathered from the fact that only twice in 261 passages did the virus become contaminated.

Freedom from Ordinary Bacteria.—Aside from the fact that the virus could purify itself in the guinea pig tissues, deliberate cultivation tests were made when it was first acquired to determine whether there might be any constant bacteria associated with this strain. Repeated cultures were attempted with the blood, with the fluid aspirated from the vesicles (the so called "lymph"), with ground infected pads and the latter filtered—all obtained on the 1st day of illness.

No constant visible microorganisms were revealed by the different methods of culture—the details of which will be described in another paper on experiments on cultivation. Hence this strain was pure, and its effects could not be ascribed to any agent cultivable by ordinary means.

The Disease in the Guinea Pig.—The clinical picture of the experimental disease in the guinea pig corresponded in the main with the comprehensive descriptions given by Waldmann and Pape, by Gins and Krause,[2] and by the members of the British Foot-and-Mouth Disease Research Committee.[3–5] Certain of the more important points are, however, worthy of recapitulation.

Method of Injection.—After a long trial with various methods of injection of the virus, we found that the method of choice, and the one by which practically all guinea pigs were infected, is the intradermal. The procedure consists in running a fine needle in the skin along the length of the hairless pads of the posterior

[2] For a general historical and descriptive account of the experimental disease in the guinea pig, see Waldmann, O., and Pape, J., *Berl. tierärztl. Woch.*, 1920, xxxvi, 519; and Gins, H. A., and Krause, C., *Ergebn. allg. Path. u. path. Anat.*, 1923–24, xx, pt. 2, 805.

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[5] Stockman, S., and Minett, F. C., *J. Comp. Path. and Therap.*, 1926, xxxix, 1.

extremities. Attached to this needle is a syringe containing the test fluid, and with pressure on the piston the skin tunnel thus made is filled as the needle is withdrawn. Three or four such injections are made on each posterior pad, and besides, the thin, loose skin along the outer and inner margins of the pad is filled until it distends to a distinct bleb-like formation. If the virus is weak or diluted, an additional inoculation is made subcutaneously in these pads. Inoculations by means of skin tunnels were also found advantageous by the workers of the British Commission.[4,5]

Injection of the virus in sites other than the pad skin was followed by specific lesions, but certainly not as regularly as those occurring after the intradermal inoculation,[6] as the following experiment illustrates.

Protocol 1.—All animals were injected with the same sample of virus taken from a guinea pig in the 1st day of illness. The infected pads of the latter were removed, ground in sand, and suspended in phosphate buffer solution (pH 7.6) to a dilution of 1:25. ½ cc. was inoculated in each guinea pig.

(*a*) Intramuscularly in the thigh. Of six animals only two showed the typical aphthæ: in one, in all four pads; in the second, in all pads and in the tongue. Incubation period 48 to 72 hours.

(*b*) Intraperitoneally. Of five animals three exhibited vesicles in all pads and in the mouth. Incubation period 3 to 4 days.

(*c*) Subcutaneous, under the abdominal skin. Of five guinea pigs, two showed aphthæ in all pads and in the tongue, one in two pads, and one in one pad. Incubation period 3 to 6 days.

(*d*) Intracutaneous in abdominal skin. Of five animals, four showed lesions in all pads and in the tongue. Incubation period 3 to 5 days.

In none of these were any lesions noted at the site of the injection.

When these methods of injection are compared with the intradermal one, already described, by which practically all of over 2000 guinea pigs were infected, it will be noted that the latter was more reliable. This test also illustrates the epitheliotropic nature of the infection, for irrespective of the site of injection, the lesions appeared only in the epithelium of the pads or of the tongue or of the mouth. Furthermore, if a proper injection of the virus was given, no natural immunity to foot-and-mouth disease could be found in guinea pigs.

[6] In this place, as in all others in which reference is made to "intradermal" inoculation, we mean to imply the particular method of injection described in the preceding paragraph.

Secondary Lesions.—As already mentioned, the Str. strain induced secondary lesions regularly. These appeared in the pads or mouth or tongue, in sites other than those injected, as early as 36 hours and as late as 5 days, but as a rule from 48 to 72 hours after inoculation. For an unequivocal determination of experimental foot-and-mouth disease in the guinea pig, the presence of these secondary lesions was essential. In doubtful instances, such as when the primary vesicles were obscured by necrosis—a condition encountered frequently in experiments with antiseptics, to be mentioned later—recourse was had to transfer to normal animals for observation of primary and secondary signs before a conclusive diagnosis could be made.

Period of Incubation.—In about 95 per cent of the cases the period of incubation was from 18 to 48 hours. In one instance, the first symptoms were noted after 12 days and in three cases, after 10 days. In the remainder, however, the primary aphthæ appeared from 3 to 6 days after injection. For these reasons, observations were prolonged to 10 to 12 days before an exact definition was obtained; and when necessary, an injection of active material was made in animals which did not react, to determine the presence of immunity. A noteworthy fact is that the period of incubation is shortened and the severity of the disease is increased as the dose or concentration of the virus is augmented.

Time of Greatest Infectivity.—Tests showed that the blood of the guinea pig was active, when withdrawn 24 and 48 hours after intradermal inoculation. On the other hand, if this blood, in a defibrinated condition, was kept at 37°C. *in vitro*, it was active for 24, but not for 48 hours. The period of greatest activity of the virus in lymph aspirated from aphthæ or in ground infected pads was obtained from lesions up to 24 hours in age. From this time to 72 hours there was a gradual diminution in virulence and after 3 days such virus was only exceptionally active. At about this latter time, the active agent, conforming to the tendency of filter-passing viruses in general, invited sometimes invasion by secondary microorganisms, followed by purulent inflammation.

Mortality.—The mortality rate from infection of guinea pigs with the Str. strain was about 1 per cent. As this rate corresponded to the normal death rate among stock animals, it may be stated that

the experimental disease induced by this strain was practically non-lethal.

Transfer to Cattle.—Finally, this strain, after propagation in the guinea pig, could induce, with ease and at will, in cattle and hogs, typical foot-and-mouth disease indistinguishable from the natural affection. The material used for such transfers was derived from guinea pigs either in the early or last passages.

Titration of the Virus.

It was essential to determine quantitatively the activity of the virus, for this estimation was important from the point of view of controlling survival or preservation, as opposed to multiplication, of the virus in subplants of cultivation tests. This information also served as a guide for interpretation of the cataphoresis tests to be reported later, and gave an idea of the relative size of the active agent.

The virus was found to be active in remarkably high dilutions as the following experiments will show:

Protocol 2.—Summary of fifteen experiments. The virus employed for these tests consisted of the aspirated lymph from 24 hour old vesicles. The diluent consisted of phosphate buffer solution at pH = 7.5.[7] Dilutions were made with pipettes. The diluted virus was injected by the intradermal method, already described, in both posterior pads of guinea pigs.

Of fifteen tests, the virus was found active in a dilution of 1:10,000,000, twice; 1:2,000,000, thrice; and 1:1,000,000, twice. In the foregoing trials, these were the final dilutions. In four additional tests where dilutions were made higher than that of the active range, in one case 1:4,000,000 was positive, but 1:8 to 32,000,000, negative; in another, 1:2,000,000 was active, but not 1:4 to 80,000,000; in a third, 1:1,000,000 was positive, but 1:2,000,000 negative; in the last case, 1:400,000 was active, but not 1:1,000,000.

All these experiments were made with the virus cleared of particles by filtration through filter paper. In the next four tests filtrations were made through Berkefeld V candles. Activity was shown in all four instances in a dilution of 1:2,000,000, the highest made.

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[7] Phosphate buffer, as mentioned here and elsewhere in these articles, is made by adding 2.5 gm. potassium acid phosphate (KH_2PO_4) to a liter of distilled water. The solution is then adjusted to the desired hydrogen ion concentration by means of potassium or sodium hydroxide. It is of utmost importance to readjust the material immediately before use, for sterilization renders it acid.

From these titrations it is to be noted that the virus diluted to 10 million times its volume may still be effective. This fact leads to several considerations, the first of which is with reference to the probable size of the active agent. Since the material is not solid with virus, but contains the diluent and various products usually found in an inflammatory exudate, the active agent is only a part of this quantity. Hence the agent should be quite minute. That the size of the virus is less than 100 mμ has also been confirmed in the study on "molecular" filtration, to be described in another paper.

Secondly, great care should be exercised in measuring or transferring virus in glass receptacles in tests for comparative activity, such as the determination of the killing effect of antiseptics, the relative value of different media in cultivation tests, etc. Spattering of the virus along the glass walls may give rise to infections which may have no bearing on the problem in hand. We have therefore substituted for test-tubes, whenever practical, chemical mixing glasses or conical beakers.

A third point is that in our experiences with cultivation of the virus, the third subplant showed activity in five different media. In this third subplant the virus was diluted by our method of seeding about 1:1,000,000. But in the fourth subplant the active agent was diluted 1:15,000,000. In this latter, however, the virus was without action. Apart from this, we had other and ample evidence to show that these media were not suitable for growth. Hence the conclusion derived from such experiments is that preservation or survival, but not multiplication is involved. Therefore, the work of Titze[8] and of others who maintained virulence only in the first few "subcultures" should be interpreted as probably indicating a mere dilution of the active agent.

Another consideration is that since one sample of the virus is active in a dilution of 1:400,000, and another in a dilution of 1:10,000,000, a factor of difference in activity between one specimen and another is 25. The investigators of the British Committee[3] have found a still greater factor to exist; one sample was inactive at 1:5000, and another active at 1:500,000. Hence, in comparative tests, such as

[8] Titze, C., *Z. Infektionskrankh. Haustiere*, 1924, xxvi, 107.

in the study of the survival of the virus in different media, trials should be made with the same specimen of virus.

Finally, the severity of the disease and the incubation period depended on the concentration of the virus: the more concentrated the virus, the more severe was the infection and the shorter the period of incubation. For example, with one sample of a 24 hour aspirated lymph virus, a dilution of 1:1000 induced the experimental disease of severe type in 24 hours in all of five guinea pigs; of 1:200,000, after 3 days, in one of two animals; of 1:2,000,000, after 4 days, mildly in one of two animals; and of 1:4,000,000, after 9 days, one vesicle was noted on the tongue of one guinea pig, of two inoculated. Higher dilutions were negative.

The rate and energy of action, therefore, are proportional to the concentration of the active agent.

Centrifugation.

Centrifugation tests were made with two objects in view: (1) to determine the centrifugability of the virus, and (2) if the virus were not centrifugable, to attempt to free, by this process, the supernatant fluid from a hypothetical body which may be the inhibiting factor in cultures, for example such bodies as fragments of tissue or cells.

As for the first object, it was determined by repeated tests that with the active guinea pig blood, aspirated lymph, or suspensions of ground infected pad tissue, centrifugation at 2500 to 3000 revolutions per minute for 2 hours did not cause the deposition of the virus: the lowest portion of the specimen was as active as the topmost layer. Thus we have confirmed the prior findings of others, notably the workers of the British Committee,[3-5] Abe,[9] and Gins.[10] However, we could not confirm thereby Frosch and Dahmen's[11] first step in cultivation, namely a concentration or sedimentation of the virus by centrifugation at 3000 revolutions per minute for $\frac{1}{2}$ to $1\frac{1}{2}$ hours.

The inability to sediment the virus may indicate that the active agent is very minute, but not necessarily that it is of the nature of a

[9] Abe, T., *Z. Infektionskrankh. Haustiere*, 1925, xxviii, 111.

[10] Gins, H. A., *Berl. tierärztl. Woch.*, 1924, xl, 661.

[11] Frosch, P., and Dahmen, H., *Berl. tierärztl. Woch.*, 1924, xl, 185, 273, 341; *Arch. wissensch. u. prakt. Thierheilk.*, 1924, li, 99.

contagium vivum fluidum. Duclaux[12] has shown that centrifugation is not an adequate means for deposition of minute particles. For theoretically with a centrifugal force 40,000 times as great as the gravity force of a particle (such as is obtained in large centrifuges) a particle measuring 10 mμ will settle only 1 cm. in 4 hours. But practically, in addition to this slow deposition due to size, the whirling current and that due to convection, since all machines develop a certain amount of heat, operate to prevent deposition. In the example cited the variations due to these currents should be multiplied by 40,000 to obtain the actual reading of the forces preventing sedimentation (Duclaux).

In respect to the removal of a hypothetical inhibiting body by centrifugation, our repeated experiences showed that the active guinea pig blood kept at 37°C. contained no virus either in the sediment or supernatant fluid, when tested 3 days after centrifugation. But at room temperature similar ground pad virus suspended in phosphate buffer at pH = 7.5, remained alive for 9 days in the sediment and for 12 days in the supernatant fluid. With aspirated lymph under the same conditions, however, both the sediment and supernatant fluid were active for at least 14 days. No further tests were made in this instance.

To complete these experiments, three additional trials were made with the sediment after showing inactivity by standing for 4 days at room temperature. This sediment was added to either fresh, or old, active supernatant fluids, and the mixture kept for from 18 hours to 3 days at 20°C. But no inhibition of the virus in the supernatant fluid was observable.

We conclude from these experiments that the hypothetical virucidal bodies cannot be deposed from suspensions of ground infected pad tissues, or of aspirated lymph, by centrifugation.

At this point, brief reference may be made to attempts to remove substances which inhibit the viability or multiplication of the virus in artificial media, by precipitation of the globulins, or inactivation of complement. For this purpose carbon dioxide was employed,

[12] Duclaux, J., Les colloïdes, Paris, 2nd edition, 1925, 84 ff.

and prolonged passage of the gas was allowed through material containing the active agent. But these tests likewise ended in failure.

SUMMARY AND CONCLUSIONS.

A strain of foot-and-mouth disease virus was recovered from a cow at the height of the disease, and was propagated through at least 261 passages in the guinea pig. Considerably over 2000 animals proved susceptible to the virus, and the virus could be transferred at will back to cattle and hogs, and then again returned to guinea pigs. No natural immunity was discovered in guinea pigs. Secondary lesions were easily and regularly induced, thus making this strain particularly favorable to experimental purposes. In general, the guinea pig may therefore be regarded as the animal of choice for laboratory studies.

The guinea pig could be infected by different methods of injection in different sites, but constant and regular production of primary and secondary lesions—or generalization of the disease—followed intradermal "tunneling" in the manner described, combined with subcutaneous inoculation of the posterior hairless pads of full grown animals. As we have indicated, the virus was peculiarly epitheliotropic, which in turn gives support to the opinion that its portal of entry may be limited.

The active agent could purify itself of chance concomitant bacteria in the first passages, in a susceptible animal—a character possessed by filter-passing viruses in general.

The virus was active in dilutions of 1:10,000,000. This shows not only the minuteness of the active agent, but also the necessity for a change of technique from that employed with larger sized infectious agents. Apart from this, the dilution factor is important in interpreting mere preservation of the virus rather than multiplication, when only early successive subplants in culture experiments are positive. Furthermore, some samples of virus were not so active—a factor of twenty-five existed between the weakest and strongest samples among fifteen titrated. This indicates that comparative tests, as, for example, of survival in different media, should be made with the same specimen. In any case, the rate and energy of action of the virus were proportional to its concentration, thus differing from the behavior of certain enzymes.

The incitant is not sedimented by centrifugation. Non-centrifugability, a property of some other filter-passing, infectious agents, is not an indication of the fluid character of the virus, as we have already explained. In view of the evidence presented and other tests to be reported later, failure of deposition is related to the minute size of the incitant. The method of centrifugation has also failed to remove "virucidal bodies" in the meaning of Frosch and Dahmen.

STUDIES ON THE PHYSICAL AND CHEMICAL PROPERTIES OF THE VIRUS OF FOOT-AND-MOUTH DISEASE.

II. Cataphoresis and Filtration.

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In the first[1] of this series of papers we described the strain of foot-and-mouth disease virus employed in our studies. We have also reported therein that guinea pigs were susceptible to the active agent in dilutions of 1:10,000,000, and that the virus was non-centrifugable. The indications pointed to a minute size of the incitant.

In the experiments to be presented in this article on cataphoresis and filtration, the findings regarding the minuteness of the incitant are confirmed and, furthermore, a relative delimitation of its size will be demonstrated. In addition, the electric charge carried by the virus will be revealed and phenomena dependent on the charge and on certain filtration reactions will be discussed.

Cataphoresis.

The electric charge carried by the active agent, as well as its isoelectric range, was a criterion by which different filtration tests could be interpreted. Its determination was useful not only to explain the remarkable resistance of the virus to certain chemicals to be described elsewhere, but also to compare the charge with that characterizing certain known microorganisms.

The main difficulty of cataphoresis experiments with this virus was the absence of a recognizable or visible particulate body. But this was overcome by adapting to our purposes the Vlès[2] apparatus.

[1] Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 673.

[2] We wish to express our gratitude to Professor Fred Vlès of the University of Strasbourg for his cordial cooperation and his invaluable assistance, not only in these experiments, but also in others on filtration.

Moreover, guinea pigs could be regularly infected with even minute quantities of the active agent, so that its attraction to one or another pole could be determined by the biological test.

The apparatus[3] employed consists essentially of horizontal U-tubes, containing in the curved arms a stoppered ampulla, and in each long arm a stop-cock. These are connected to communicating cups by curved syphons which are joined to each other by a transverse tube carrying also a stop-cock. The latter allows a prior establishment of a hydrostatic equilibrium. The orifices of the curved syphons are plugged with moist cotton to prevent hydraulic disturbances. The tubes, fixed to a board, are placed in series of four (because four determinations at different hydrogen ion concentrations were made at one time). At either end of the series, union with the house current is made through a solution of cupric sulfate, and in the connecting cups through physiological saline solution (0.9 per cent at pH = 8). In the syphons of each tube of the series is placed the phosphate buffer[4] at the hydrogen ion concentration at which the virus is tested. In the ampullæ is added the virus, diluted with phosphate buffer at different pH readings, thus filling completely the respective U-tubes to the stop-cocks.

In the following experiments the difference of potential at the limits of the series in an open circuit was 220 volts. A milliamperemeter was then placed in the circuit, which registered from 0.8 to 4.7 ma. for the four tubes, and the fall of potential for each tube was from 20 to 50 volts. The time of cataphoresis lasted from 20 to 70 minutes. We have found that the best result was obtained when the virus was filtered and diluted 1:1000 in phosphate buffer, at a milliamperage of 0.8, with a fall of potential of 20 to 30 volts and cataphoresis for 70 minutes.

This experiment, one of a series of ten, is illustrative:

Protocol 1.—24 hour aspirated lymph virus was diluted 1:1000 with phosphate buffer at pH = 7.5, and filtered through Berkefeld V candles. This material, on injection intradermally in the pads of guinea pigs, showed activity in each of graded dilutions from 1:2000 to 1:200,000.

The substance was then separated into four portions and each was adjusted to different pH readings by the colorimetric method. At the same time phosphate

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[3] Vlès, F., and de Coulon, A., *Arch. physiq. biol.*, 1924, iv, 43.

[4] Phosphate buffer is made by adding 2.5 gm. potassium acid phosphate (KH_2PO_4) to a liter of distilled water. This is then adjusted to the desired pH by means of potassium hydroxide. It is of utmost importance to readjust the materail just before use, for sterilization renders it acid.

buffer adjusted to similar degrees was prepared. At the termination of the cataphoresis experiment the following electrometric pH measures were obtained:

Tube	Buffer	Virus	Average buffer and virus
1	6.63	6.84	6.73
2	7.68	7.62	7.65
3	8.00	7.90	7.95
4	8.17	8.05	8.11

The cataphoresis was continued for 70 minutes with a fall in potential of 20 to 30 volts with a milliamperage of 0.8.

Guinea pigs were then injected intradermally in the pads with the material removed from the negative and positive poles of each of four differently adjusted specimens with these results:

pH = 6.73
Negative pole: 2 guinea pigs; both positive after 48 hours.
Positive pole: 2 guinea pigs; both negative.

pH = 7.65
Negative pole, 1:2000: 2 guinea pigs; both positive after 48 hours.
1:20,000: 2 guinea pigs; both positive after 48 hours.
Positive pole, 1:2000: 2 guinea pigs; both negative.
1:20,000: 2 guinea pigs; both negative.

pH = 7.95
Negative pole, 1:2000: 2 guinea pigs; both positive after 48 hours.
1:20,000: 2 guinea pigs; both positive after 48 hours.
Positive pole, 1:2000: 2 guinea pigs; both negative.
1:20,000: 2 guinea pigs; both negative.

pH = 8.11
Negative pole: 2 guinea pigs; both negative.
Positive pole: 2 guinea pigs; both positive after 48 hours.

Results of other tests at pH readings different from those mentioned showed:

pH = 6.6
Negative pole, 1:500: 2 guinea pigs; both positive after 48 hours.
Positive pole, 1:500: 2 guinea pigs; both negative.

pH = 7.0
Negative pole, 1:500: 2 guinea pigs; both positive after 48 hours.
Positive pole, 1:500: 2 guinea pigs; both negative.

pH = 7.4
Negative pole, 1:500 to 1:100,000: in all dilutions, all guinea pigs positive after 48 hours.
Positive pole, 1:500 to 1:100,000: in all dilutions, all guinea pigs negative.

pH = 7.5

Negative pole, 1:250 to 1:250,000: in all dilutions, all guinea pigs positive after 2 to 4 days.

Positive pole, 1:250 to 1:250,000: in all dilutions, all guinea pigs negative.

pH = 8.0

Negative pole, 1:150 to 1:30,000: in all dilutions, all guinea pigs positive after 48 to 72 hours.

Positive pole, 1:150 to 1:30,000: in all dilutions, all guinea pigs positive after 24 to 48 hours.

pH = 8.5

Negative pole, 1:500: 2 guinea pigs; both negative.

Positive pole, 1:500: 2 guinea pigs; both positive after 48 hours.

It appears, therefore, that the foot-and-mouth disease virus is capable of wandering in an electric field and then, if the active agent is at a hydrogen ion concentration below pH = 8, the direction of motion is toward the negative pole (electropositive); if above pH = 8, toward the positive pole (electronegative). At about pH = 8, there is a tendency to move in either direction (isoelectric range). Hence it may be stated that under ordinary conditions, the active agent has an electropositive charge, and that its isoelectric range is at pH = about 8. It should be remembered that, in view of the absence of a visible particulate body, and the slight changes in hydrogen ion concentration during cataphoresis, this figure is relative; hence the term "range" is employed rather than the word "point."

It was noted that, as a rule, there was clouding at the pole opposite to that to which the virus was attracted. For example, if the fluid at the positive pole appeared hazy, and at the negative limpid, then the material at the negative pole contained the active agent. Whether the force of the electric current is sufficient to tear the virus from the electronegative protein particles to which it may be attached,[5] we are unable to say, but these findings are suggestive. The possible objection that the virus wanders along with protein does not hold, for the virus moved in a different direction from the ordinarily electronegative proteins.

The fact that the virus has an unusually high isoelectric range and

[5] In another section will be described the adsorption of the virus to large protein coagula.

is, under ordinary conditions, electropositive does not speak against its possible protein or living nature. For, although bacteria are generally electronegative in charge, spirochetes, as a genus, carry electropositive charges (with the exception of *Treponema pallidum*); so do trypanosomes, such as the species of *brucei, equiperdum, gambiense*, and *rhodesiense*. Among protein substances, fibrin and gliadin (at pH = 9.3) are known to possess a high isoelectric point. The outstanding fact is that the virus, in respect to its charge, is different from ordinary bacteria.

The determination of the charge, however, was of greatest importance in interpreting the results of ordinary and "molecular" filtration experiments, and therefore gave indirectly some indication of the relative dimensions of the causal agent.

Filtration.

The filters employed in the following experiments comprised the Seitz' asbestos discs, Berkefeld V and N candles, Chamberland's L 1, L 2, L 3, L 5 (F), L 7 (B), L 9, L 11, and L 13 bougies, various thicknesses of collodion membranes, and, finally, Bechhold's ultrafilter prepared with different percentages of acetic collodion.

It is important to note that the foot-and-mouth disease virus, as employed in the following tests, may contain the incitant in a free state, as well as adsorbed to particles. That the virus can be free and that there is a lack of complete adsorption to a number of different substances has been observed by several investigators (Dahmen, Gins, Bedson, Burbury and Maitland, and Stockman and Minett). Furthermore, there is practically no loss in activity of the virus after filtration through coarse and fine filters. In addition, filtrations proceed in a definite, regular manner. For these reasons, and from the results of cataphoresis experiments showing a separation of the virus from protein particles, one cannot regard the whole of the incitant as adsorbed to minute particles of the same size.

Experience with the Seitz' filter showed that the virus filtered easily through one disc of asbestos. We have not employed this method for study of filtration phenomena, but merely to purify the

virus from contaminating microorganisms. For this purpose, the Seitz' filter is quite effective.[6]

In regard to the other methods of filtration, a general principle was involved in all cases, namely, all the filters carry an electronegative charge. As the virus has an electropositive charge, considerable adsorption occurs during the passage of the active agent through the candles, bougies, or membranes. In some filters in which the pore size is minutely small, and the charge strongly negative, complete adsorption takes place with the consequence that the filtrates contain no virus.

Filtration through Berkefeld V and N Candles.—The material for these tests consisted of aspirated lymph, or of ground infected pads suspended in phosphate buffer at pH = 7.5 or 7.6. In any event, preliminary filtration through one layer of filter paper was resorted to. This was done to prevent clogging of the small candles with particles of tissue, fibrin, or cells.

As tests of the charge and relative pore size of these filters, there were passed through them suspensions containing particles of either charge and of different sizes. Electropositive micellæ, such as methylene blue, basic fuchsin, and night blue regularly failed to pass, but electronegative particles, such as acid eosin and Prussian blue, readily traversed the candles. Hence these filters carried an electronegative charge.

It was therefore necessary, in determining the size of particles capable of passing through the V and N filters, to eliminate adsorption due to filtering oppositely charged substances. Accordingly, collargol, the mean diameter of its micellæ being 20 mμ and colloidal arsenic trisulfide, 100 mμ, were tested. Both passed rapidly through V, and definitely, but less speedily, through N candles. Both substances have an electronegative charge. Hence these Berkefeld filters, with the adsorption action counteracted, allow the passage of particles of at least 100 mμ in diameter. This agrees in part with the determination of the size of the intergranular spaces by Bechhold's formula,

[6] For more extensive studies on this type of filter, the reports of the investigators of the British Committee should be consulted. References are cited in the first paper of this series.

as given by Mudd.[7] The latter found the diameter of the V type to be 0.38 μ (380 mμ), and of the N type, 0.45 μ (450 mμ).

In respect to the virus of foot-and-mouth disease, a standard substance for comparison should be one with a positive charge, and of a definite known size. For this purpose, pure, crystallized hemoglobin in solution, and therefore molecular, was employed. Its isoelectric range is at pH = 6.75, and the size of its molecule, as computed by Bechhold,[8] is 3.6 mμ in diameter. Suspended in phosphate buffer (employed also in suspensions of the virus) at pH = 6, hemoglobin is electropositive, and at pH = 7.5, electronegative. In the former condition this substance passes through V and N candles; in an electronegative condition, the passage is more free and completely unobstructed. Since the virus with an electropositive charge also passes regularly through both these filters, showing at times activity in at least a 1:2,000,000 dilution of the filtrate, the conclusion derived from these observations is that the active agent is at least larger than the molecule of hemoglobin. This has been confirmed by our experiences with filtrations through Chamberland bougies.

Filtration through Chamberland Bougies.—The Chamberland bougies of all types are similar to the Berkefeld candles in that they are charged electronegatively. For, electropositive particles, such as methylene blue, basic fuchsin, and night blue dye do not pass through their walls, but the electronegative micellæ of acid eosin, Prussian blue, collargol, and colloidal arsenic trisulfide do. In regard to hemoglobin, at pH = 6, with an electropositive charge, this substance passes these filters only slightly—the first few drops of filtrate are clear but after a time traces of hemoglobin are revealed by spectroscopic examinations. At pH = 7.5, with an electronegative charge, hemoglobin traverses freely all the types of Chamberland filters. In other words, as an electropositively charged molecule, hemoglobin first satisfies the avidity of the electronegative material of the bougie and later its minute size enables it to pass through the barrier.

A similar experience was obtained with the electropositive virus. With bougies of graded series to L 5, the active agent traversed their

[7] Mudd, S., *J. Bact.*, 1923, viii, 459.

[8] Bechhold, H., *Z. physik. Chem.*, 1908, lxiv, 328.

walls. But with more dense types, the passage became more difficult, until with the L 11 type,[9] the filtrates were free from virus. For example, five trials with five different new L 7 type bougies yielded in only one case a positive filtrate; three filtrations through three different, new L 9 type bougies, resulted in one positive; and seven tests with seven different L 11 type bougies, six of which were new, the seventh having been used more than once, failed in all instances. In these experiments the virus was suspended in phosphate buffer at pH = 7.5 (electropositive). Diluted in similar material at pH = 8.5, the virus, then in an electronegative state, passed freely through these bougies.

The pore size of the L 11 type of Chamberland filter has been determined after Bechhold's[8] air pressure, revised method, as 110 to 150 mμ. It appears, therefore, that the foot-and-mouth disease virus can, by elimination of adsorption due to opposite electric charges, traverse these small spaces. On the other hand, if adsorption due to opposite charges is unhindered, the virus fails to pass. But, in the latter relation, hemoglobin under similar conditions can traverse this filter. Hence the active agent is larger than the hemoglobin molecule of 3.6 mμ diameter. Experiments with filtration through collodion membranes, to be described immediately, confirm this relative measurement.

Filtration through Collodion Membranes.—For a determination which might give a measurement of the virus between narrower limits, and for a more extended study of the filtration phenomena, a still tighter filter was employed, namely, the collodion membrane.

Collodion membranes are negatively charged. Indeed, the individual particles of pure collodion remain so charged, or become even more strongly negative, in the presence of either acid or alkali, as measured by the cataphoretic potential difference between these particles and water (Loeb[10]).

The collodion membranes, usually in the form of small sacs, were

[9] Several trials with a number of the L 13 type bougie, supposed to be the tightest of all of the series, resulted in irregular filtrations of titrating reagents. Hence work with this type was discontinued.

[10] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 2nd edition, 1924, 79 ff.

prepared with French Codex Collodion (5 per cent). In all 68 sacs were made with different proportions and conditions of collodion, so that eighteen different experiments were performed. The concentration of colloidion varied from 5 per cent to 12 per cent (the 12 per cent corresponding to 15 seconds reading on the apparatus devised by Gates,[11] for measuring its relative viscosity). From one to three layers were used, and dipping in 95 per cent alcohol was resorted to in some instances to increase the permeability of the sacs. Negative pressure from 10 to 25 cm. of mercury was applied. The virus comprised 24 hour aspirated lymph, diluted 1:50 or 1:100 in phosphate buffer at pH = 7.5 or 7.6 to pH = 8.5 which in all tests, as measured by control inoculations in guinea pigs, was quite active. Six experiments were also made with sacs prepared after the manner of Levaditi, Nicolau, and Galloway,[12] who used three layers of 5 per cent collodion (Codex), without alcohol immersion.

To summarize the results of these experiments: We found that the collodion membranes prepared in the manner stated are not suitable for the filtration of the virus of foot-and-mouth disease. For more than half of the number of membranes showed macroscopic holes before put to test. In the remaining ones, we could not convince ourselves that microscopic holes were absent. In the absence of standardization of the membrane, it was necessary that each sac be tested separately by titration with known substances of definite charge and size. Otherwise the results were valueless. Membranes which we thought hole-proof and at the same time made so as to withstand a negative pressure of from 10 to 25 cm. mercury (without this negative pressure, filtration was impossible), failed, as a rule, to allow passage of methylene blue, eosin, collargol, hemoglobin, etc.—the materials employed for titration of the Berkefeld V and N and all types of Chamberland filters. They did allow the passage of peroxidase (which we found to be more easily filterable than blue litmus), of blue litmus, the size of which is computed by Bechhold[8] to be 1.8 mμ in diameter, and of crystalloids. The crystalloids which traversed the membranes were, in order of molecular weights from

[11] Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 25.

[12] Levaditi, C., Nicolau, S., and Galloway, I.-A., *Compt. rend. Acad.*, 1926, clxxxii, 247.

strychnine, molecular weight, 397; acid quinine sulfate, to basic quinine sulfate, molecular weight, 890.[13] The virus, on the other hand, failed to pass through these membranes.

It may therefore be concluded that collodion membranes of the types mentioned are permeable to colloids containing particles in size of the order of blue litmus, and to crystalloids, but not to the virus carrying positive or negative charges. Furthermore, in the absence of standardized membranes, they are unsuitable for titration experiments. We have, however, confirmed the observation of Stockman and Minett[14] that the virus does not traverse two layers of 5 per cent collodion. One conclusion may be derived from these experiments: the active agent is not comparable to crystalloids in respect to filtration through collodion membranes.

It was then found necessary to seek a more permeable membrane, by which one could delimit the size of the virus more precisely and, at the same time, be free from the variables in the methods already mentioned. For these purposes we found the Bechhold ultrafilter membranes quite satisfactory.

Filtration through the Bechhold Ultrafilter Membranes.[15]—The prepared Bechhold discs are made by impregnating in a vacuum Schleicher and Schüll filter paper with different concentrations, from $1\frac{1}{2}$ to $7\frac{1}{2}$ per cent, of acetic collodion. By this method unevenness of the layer and microscopic holes are eliminated; the membrane is strong enough to withstand the necessary negative pressure and a concentration of as little as $1\frac{1}{2}$ per cent of the collodion can be used with, as a rule, unequivocal results.

We adapted these discs to our purposes by cutting each membrane to a circumference corresponding to that of the lower part of a Seitz (Manteufel,[16] E. K. model) filter. The membrane was placed over the wire gauze support and a smooth, well fitting, firm, rubber washer

[13] Strychnine was tested for its presence in the filtrates by inoculation of frogs; quinine, by opalescence, in a strong beam of light, after acidulation with sulfuric acid.

[14] Stockman, S., and Minett, F. C., *J. Comp. Path. and Therap.*, 1926, xxxix, 1.

[15] The membranes are manufactured by Schleicher and Schüll, Düren, Rhineland.

[16] Manteufel, P., *Centr. Bakt., 1. Abt., Orig.*, 1924, xciii, suppl., 259.

was superposed. The cup end was then tightly screwed in and the mechanism was tested for leakage at the negative pressure employed in each test.

The Bechhold disc is electronegative. To eliminate the adsorption of substances oppositely charged, both the virus and the test materials for titration were employed in a condition of the same, that is, negative charge. Hence, for virus, we prepared 24 hour old lymph as-

TABLE I.

Filtration through Bechhold's Ultrafilter Membranes.

Substance	Charge	Size of particle	Per cent acetic collodion in membrane															
			7½ per cent		6 per cent		4½ per cent		3 per cent					1½ per cent				
			Number of membranes employed	Results	Number of membranes employed	Results	Number of membranes employed	Results	Number of membranes employed	Results in each case. Membrane No.				Number of membranes employed	Results in each case. Membrane No.			
										1	2	3	4		1	2	3	4
Virus	Neg.	X	4	−	4	−	4	−	4	+	−	−	−	4	+	+	+	+
Blue litmus	Neg.	1.8mμ	4	+	4	+	4	+	4	+	+	+	+	4	+	+	+	+
Hemoglobin 1 per cent	Neg.	3.6mμ	4	−	4	−	4	−	4	+	+	+	−	4	+	+	+	+
Collargol	Neg.	20mμ	4	−	4	−	4	−	4	+	−	−	−	4	+	+	+	+
Colloidal arsenic trisulfide	Neg.	100mμ	4	−	4	−	4	−	4	−	−	−	−	4	+	−	−	−

− indicates the non-filterability and + the filterability of the substance tested.

With the 4½ to 7½ per cent collodion membranes, the results were uniform. With the 1½ and 3 per cent membranes, there were slight variations, so that the tests with each are tabulated.

pirated from fresh, unbroken vesicles, diluted to 1:100 in phosphate buffer at pH = 8.5. The substances for titration consisted of blue litmus and hemoglobin, each suspended in the phosphate buffer at the same hydrogen ion concentration. The collargol and the colloidal arsenic trisulfide, both electronegative, were prepared fresh after the directions of Ostwald.[17] As a test for the charge of the membrane

[17] Ostwald, W., Manipulations de chemie colloidale; translated by Veillinger, E., from the 4th German edition, Paris, 1924.

and its ability to adsorb an oppositely charged substance, a suspension of electropositive night blue dye (*bleu de nuit*) was used. Complete adsorption of this dye occurred in every case.

In Table I are summarized the experiments made with membranes of different permeability, that is, of different thicknesses, containing from $1\frac{1}{2}$ to $7\frac{1}{2}$ per cent acetic collodion. The virus under electronegative conditions is compared with the electronegative test substances, blue litmus, hemoglobin, collargol, and colloidal arsenic trisulfide. These varied in size from 1.8 mμ to 100 mμ in diameter. The negative pressure required for each type of membrane differed with the thickness of the layer. For example, with the $7\frac{1}{2}$ per cent membrane, -30 cm. mercury pressure was employed; with the 6 per cent, -10 cm.; with the $4\frac{1}{2}$ per cent, from -5 to -10 cm.; with the 3 per cent, -5 cm.; with the $1\frac{1}{2}$ per cent, -5 cm. In all cases the filtrates were tested for the presence of virus by intradermal inoculation of the hairless pads of guinea pigs; and for the titrating substances by colorimetric, spectroscopic, and chemical tests. It may be stated here that the virus taken from the apparatus, before filtration and at pH = 8.5, was injected intradermally in three to four guinea pigs in each instance. After 24 hours all animals showed the typical experimental disease.[18]

An examination of the tabulated, summarized results reveals that, in general, there are only a few irregularities in filtration through the single layer Bechhold membranes—indeed, fewer than were expected. But in view of the fact that each membrane was titrated and its porosity delimited, the results of "molecular" filtrations appear to be conclusive. Furthermore, the selective passage of the titrating substances which we have employed is identical to that reported by Bechhold.[8]

Only the minute particles of blue litmus could traverse the $4\frac{1}{2}$ to $7\frac{1}{2}$ per cent membranes and then quite regularly. The virus uniformly failed to pass. The virus did, however, traverse one of four membranes of the 3 per cent type. In that instance collargol also passed. In the three others of this series, two allowed the passage of hemo-

[18] In these, as in all filtrations, only from 5 to 10 cc. of material was filtered, and the time regulated. Results therefore cannot be ascribed to employment of large quantities, filtered over a long period.

globin and one the blue litmus only. On the other hand, the virus filtered uniformly through all membranes of the 1½ per cent type. In these the porosity was of such an order of magnitude that in all collargol traversed the membranes as well, and in one case, the colloidal arsenic trisulfide.

One may conclude, therefore, that the order of magnitude of the active agent of foot-and-mouth disease, after elimination of interference by adsorption due to unlike charge, is, in relative terms, correspondingly between that of collargol and of colloidal arsenic trisulfide particles. In other words, the size of the virus is relatively between 20 and 100 mμ.[19]

These tests have not only served to delimit the size of the active agent, but also serve to refute the notion that the virus is a fluid or a *contagium vivum fluidum*, after the meaning of Beijerinck,[20] of which more will be said later. Furthermore, the same comparative ease with which the virus and collargol particles filtered through the 1½ per cent membranes and the difficulty with which colloidal arsenic trisulfide traversed similar discs indicate that the size of the active agent may be nearer the minimal reading than the maximal. The minuteness of size may explain the invisibility of the virus; the difficulty of its artificial cultivation and its resistance to alcohol and other chemicals, to be described in another paper; its non-centrifugability; and its activity in very high dilutions. For particles of these relative dimensions are governed by different physical laws than those influencing larger structures.[21] Finally, the question arises as to whether these measurements preclude the notion that the active agent is living. In the absence of a standard for comparison, we may compare the virus to bacteria in respect to their chief function, the enzymatic, and their composition, which is mainly protein. We have determined (*vide supra*) that peroxidase can pass freely through spaces which do not permit the passage of litmus, the particles of which are

[19] These tests supply a basis of only a relative calculation. Hence it was decided, especially in view of the definite limitation of time for experimental work in Europe, to forego further trials in an attempt to narrow the range of size to closer approximations.

[20] Beijerinck, M. W., *Centr. Bakt., 2. Abt.*, 1899, v, 27.

[21] Vlès, F., *Rev. scient.*, 1921, lix, 294.

estimated at 1.8 mμ in size (Bechhold). On the other hand, assuming the virus to be spherical and of a diameter of 50 mμ, it should be more than 2500 times the volume ($V = \frac{4}{3}\pi r^3$) of the protein molecule hemoglobin. Furthermore, Vlès[22] has calculated that spherical bodies of the order of magnitude of 30 mμ may contain 360 protein molecules, and those of 90 mμ, 9000.

SUMMARY AND CONCLUSIONS.

Cataphoresis experiments show that the virus of foot-and-mouth disease carries, under ordinary conditions, an electropositive charge. Its isoelectric range is at the high point of pH = about 8. Although cultivable bacteria are, as a rule, electronegative, yet certain protozoa, such as trypanosomes and spirochetes are also electropositive. In respect to charge, then, the virus is different from ordinary bacteria, but there is nothing in this finding to indicate an inanimate character of the incitant. A knowledge of the charge, however, aids in the interpretation of certain filtration phenomena, and indirectly in delimiting the size of the virus. In addition, it serves to explain its remarkable resistance to certain chemicals—a subject to be dealt with in the next paper of this series. Finally, cataphoresis indicates the possible separation of the virus from protein particles.

Filtration experiments were made with different types of filters: Seitz, Berkefeld V and N, and Chamberland, of practically all sizes, collodion membranes, and Bechhold's ultrafilter. The results confirm the electropositive charge of the virus, as well as the minuteness of its size. Filtration was effected through the Seitz, Berkefeld, and Chamberland filters. In regard to the latter, the active agent passed through the L 11 only when its charge was shifted to negative: under ordinary conditions, carrying an electropositive charge, it failed to traverse this more dense wall, and was completely adsorbed in the oppositely charged barrier. Filtrations through electronegative collodion membranes, prepared in different ways and of varying thicknesses, resulted, as a rule, in failure, unless the thinnest and most permeable membranes were employed. But in these, the complication of microscopic holes was to be considered. Hence this method

[22] Personal communication.

was regarded as impracticable. Success, however, was obtained with Bechhold's ultrafilter membranes of the most permeable type, and with these it was possible to measure relatively, by a system of "molecular" filtrations, the size of the incitant. This was found to be, in relation to other particles of like charge, between 20 and 100 mμ in diameter.

The filtration phenomena of the foot-and-mouth disease virus can be accounted for on the basis of the minute size of the particles of the incitant carrying an electropositive charge, and no evidence can be deduced therefrom that the virus is of a fluid character. For the relative size of the particles is constant and the invariability of the limits of measurement contradicts the notion that the incitant may be a "solute" varying in dimensions in different "solvents."

STUDIES ON COMPLEMENT FIXATION IN TUBERCULOSIS. III.

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In the course of the work recorded in two previous papers (1, 2) on this subject, increasing interest has developed in a substance extractable from dried tubercle bacilli by hot alcohol, which serves as an antigen in the complement fixation reaction. Much work on the antigenic constituents of the tubercle bacillus and their chemical characterization has appeared recently. Wadsworth, Maltaner and Maltaner (3) have increased our knowledge of the lipoidal constituents and have used them in practical diagnostic work. The experience of these authors is in entire accord with my own in that, in so far as could be determined with the immune serum available to them, bacillary products when subjected to *prolonged* extraction with lipoid solvents, are deprived of residual antigenic activity. Others, particularly Dienes and Schoenheit (4, 5), while recognizing the presence and importance of the constituents extractable by the lipoid solvents, laid stress on the fact that with their sera and antigenic preparations, antigens residing with the protein fractions are also demonstrable and that some sera react with the lipoidal antigens, others with the protein. In no case, however, is it clear that their protein preparations are free from substances extractable with hot alcohol.

The work of Zinsser and Mueller (6) and of Petroff (7) emphasized the importance of "residue" after precipitation of protein-containing extracts with heat and acetic acid. These observations brought the work on the tubercle bacillus into closest conjunction with the work of Avery and Heidelberger (8) on the specifically precipitable substances derived from the pneumococcus, the active substances in the latter case being carbohydrates.

Laidlaw and Dudley (9), working with the tubercle bacillus, have separated a carbohydrate material reacting with a complement-fixing serum in dilution of 1/6,000,000 from residue remaining after prolonged contact of the bacilli with alcohol and chloroform. This substance is said to be insoluble in alcohol, but whether insoluble in hot alcohol is not stated. In many respects the substance is analogous to those obtained from the pneumococcus by Avery and Heidelberger. The possibility remains open that this substance may be found associated with either the proteins or the lipoidal fractions of the tubercle bacillus, according to the circumstances of preparation.

This possibility is emphasized by the observations of Dienes and Schoenheit (10), who have returned to the examination of the lipoidal fraction and have obtained an alcohol-soluble substance, which after a preliminary purification, reacted in the complement fixation reaction in a dilution of 1/2,000,000. The alcoholic extract in the less advanced stages of purification contained carbohydrate to the extent of 26 per cent in some instances. The carbohydrate content of the more highly purified material was not determined.

The determination of the identity or otherwise of the carbohydrates of Laidlaw and Dudley with the alcohol-soluble fraction giving carbohydrate reactions as observed by Dienes and Schoenheit, is likely also to settle finally the much debated question of whether the antigenic relationships of the tubercle bacillus to the sera of animals either naturally diseased or injected one or more times with entire tubercle bacilli, are single or multiple. Till now, it may be repeated, there is no satisfactory evidence that these more natural sera react in complement fixation with any residual material after complete extraction with hot alcohol. It is conceivable, however, that such severe extraction may injure the antigenic qualities of the residue. It is also more than probable that potentially antigenic substances may remain in completely extracted residues and will be made evident by appropriate immunological procedure even though they do not react with sera prepared with the whole bacilli.

I am now able to record a simple method for the extraction, concentration and preliminary purification of the fraction soluble in hot ethyl alcohol. The procedure we have followed should serve as a most convenient starting point for further chemical manipulation of

this portion of the lipoidal fraction. I have also found that normal serum under certain conditions gives a precipitation reaction with the material. This reaction is interesting in itself and its recognition as a possible source of confusion with true immunity reactions may be important.

EXPERIMENTAL.

The work here recorded was all done with a rapidly growing bovine type culture of low virulence (Bovine III). Dried bacilli are extracted repeatedly in a Soxhlet extractor with absolute ethyl alcohol. When the extracts are cooled, a white precipitate forms, which is largely, although not completely, redissolved when the alcohol is again boiled. The extracts from a number of operations may be combined, boiled, filtered through paper while hot, then chilled and filtered in the ice box, giving a considerable mass of whitish paste which when quite dry can easily be ground to a powder. One or two reprecipitations under these conditions result in a powder which is completely soluble in hot alcohol to a practically colorless solution.[1] The experiments to be reported were carried out with material at this stage of purification. Weighed quantities were dissolved in hot alcohol and dilutions made from such a stock solution.

If a 0.5 to 1.0 per cent solution of this material be brought to a boil and measured with a warm pipette into 0.85 per cent NaCl solution at the temperature of the room, the 1/10 dilution is opalescent, with a bluish tinge. These solutions, or suspensions, allowed to stand for days at room or refrigerator temperature usually show no precipitation.

Such dilutions in normal saline solution freshly prepared, have been the starting point for the use of the substance in the fixation and precipitation reactions herein presented. When higher dilutions are desired, these are made by using a less concentrated alcoholic solution as a starting point, or a lesser amount of the alcoholic solution. Dilutions in series of the saline suspensions have not been practiced.

The method employed for the fixation test was fully described in our second publication. Essentially, the primary incubation is 2 hours at 37–38°, in the water bath; from 2 to 2½ units of guinea pig complement are used; approximately 10 units of hemolytic amboceptor are employed; the second incubation is ½ hour; and finally, an excess of immune serum, amounting to from 2 to 10 times the fixation unit, has been used.

When, as in this case, the antigenic value of a preparation is under consideration, the experiment may be variously interpreted. The use

[1] I am indebted to Dr. P. A. Levene for his active interest in guiding me to this simple procedure for preliminary purification of the material.

of a definite excess of complement and a large excess of hemolytic amboceptor as outlined above made our conditions very rigid. In the same sense we have considered the end-point to be at the limit of complete fixation. Dilutions of antigen giving partial fixation have been left out of account. Under these conditions the antigenic value of an ordinary sample of the material prepared as above described tested against an immune goat serum was in the neighborhood of 1/1,300,000 dilution calculated on the weight of the powder. Reading partial fixations or otherwise relaxing the conditions of the test would make it possible to recognize the presence of the antigenic substance in two- or possibly fourfold greater dilution than this, but probably a certain degree of accuracy would be lost to comparative experiments.

As an antigen this substance has advantages in the conduct of the fixation reaction. It seems to be very stable when suspended in alcohol and if these suspensions are protected from evaporation, the preparation can be regarded as a standard one, at least for a number of months. Repeated momentary re-solution by boiling, with care to limit evaporation, does not cause deterioration to an appreciable extent. The fixation unit is usually approximately 1/100 of the anti-complementary amount. 2 fixation units give a maximum effect.

Allowing for difference in technique, it would appear that this extract in hot alcohol once or twice reprecipitated by chilling out, approaches in activity the best preparations of Dienes and Schoenheit. It is also of the order of activity of the carbohydrate of Laidlaw and Dudley. This activity together with the previously determined fact that when this substance is completely removed the bacillary bodies no longer react in the complement fixation test, seem to warrant further attention to it.

In connection with fixation studies, Calmette (11) and Caulfeild (12) have repeatedly made reference to another phenomenon spoken of as the inhibition of fixation. For the demonstration as carried out by Caulfeild quantities of the specific antigen in themselves sufficient to be anticomplementary are mixed with the immune serum and incubated with the complement. If the serum is active in the inhibitory sense hemolysis will take place when the hemolytic system is completed. This is paradoxical in that either the antigen alone, or

the antigen + serum would be expected to fix or inactivate the complement.

With the antigen preparation under discussion and sera available during the past year, I made an extra effort to demonstrate the phenomenon of inhibition. The inhibition reaction has sometimes been in evidence but the experiments were marked by many irregularities and I reached no final conclusion as to their significance. The nature of the irregularities were such as to suggest that smaller serum quantities might possibly permit the demonstration where larger quantities would fail. The time factor might also be of decisive importance. I was thus led to set up unusually long dilution series and accidentally to the interesting observation that normal serum gives a precipitation reaction with the antigen under rather unusual conditions.

The experiment fully developed after many tentative trials is as follows:

The stock alcoholic solution (½ per cent) of the antigen as above described, is brought to a boil and an appropriate quantity is added to 20 volumes of 0.85 per cent NaCl solution. Of this 0.9 cc. portions are put in a series of agglutination tubes. A series of serum dilutions is prepared such that when 0.1 cc. or less of each is added to the antigen in the tubes, the serum amounts will range from 0.01 cc. to 0.000002 cc. in a total volume of approximately 1 cc. The tubes are shaken and put in the ice box. The result is the formation of an abundant flocculent precipitate in a few tubes. Tubes adjacent in either direction to those showing precipitation, show an increased turbidity without the formation of flocculi. An essential condition is that the serum used shall have previously been heated to 56°C. for ½ hour or longer. "Fresh" beef serum gives an increased turbidity without flocculation over a much wider zone. The combined results of a number of experiments with beef serum in which the above conditions were fulfilled, although at different times, are shown in Table I.

It will be noted that the precipitation centers at about concentration 0.00007, and that the turbidity change without precipitation is irregularly extended in either direction from the precipitated tubes. Serum 1116 (9-I-f) shown in Column 7 is unheated serum showing the turbidity change only.

Of the serum samples shown in this table, two numbers, 1162 and 1163, are normal, three, 1113, 1116 (13-XI) and 1118 (28-X), were from animals treated previously with avirulent bovine type bacilli,

and showing at this time moderate values in the complement fixation test. The precipitation values all approximate those of the normal animals.

TABLE I.

Serum sample*†	1163 (28-I)	1162 (28-I)	1113 (9-I)	1118 (28-X)	1116 (13-XI)	1116 (9-I-f)	1118 (26-I)
Serum amount *cc.*							
0.01	0‡	0	0	0	0	0	0
0.008	0	0	0	0	0	0	0
0.006	0	0	0	0	0	T	0
0.004	0	0	0	0	0	T	0
0.002	0	0	0	0	0	T	0
0.001	0	0	0	0	0	T	0
0.0008	0	0	0	0	0	T	0
0.0006	0	0	T	T	0	T	0
0.0004	T	0	T	T	0	T	T
0.0002	T	+++	++++	++++	T	T	T
0.0001	++++	++++	++++	++++	++++	T	++++
0.00008	++++	++++	++++	++++	++++	T	++++
0.00006	++++	++++	++++	++++	++++	T	++++
0.00004	++++	++++	++++	++++	+++	T	++++
0.00002	++++	++++	T	T	T	T	++++
0.00001	0	0	T	T	T	0	0
0.000008	0	0	T	T	0	0	0
0.000006	0	0	0	T	0	0	0
0.000004	0	0	0	0	0	0	0
0.000002	0	0	0	0	0	0	0
0.000001	0	0	0	0	0	0	0

Alcoholic extract of tubercle bacilli L. 1. 0.5 per cent hot alcoholic solution diluted 1/19 with normal salt solution. Of this 0.9 cc. used in test. Serum dilutions added to total volume 1 cc. Readings after 24 hours in ice box.

* 1162 and 1163, normal calves. 1113, 1118 (28-X), 1116, calves treated with living avirulent tubercle bacilli. 1116 (9-I-f) serum not heated. 1118 (2601) calf 4 weeks after infection with virulent culture of tubercle bacillus; died 2 weeks later.

† Except 1116 (9-I-f) all sera heated to 57–58°C. for ½ hour.

‡ ++++ = complete precipitation. T = increase in turbidity without precipitation. 0 = no change in fluid.

The reaction may be developed at either 37° or at 56°C., but it is not materially hastened or more delicate at these temperatures. If the completed reaction series is placed at 56°, the flocculi contract and

the precipitate becomes heavy almost at once, but precipitation does not occur in tubes where it was not previously present. Sometimes the reaction is not fully developed in less than 48 hours.

Following Calmette's method for the separation of his inhibitory substance from the fixing substances in immune serum, serum diluted

TABLE II.

Serum	1122 (26-I)	1122 (26-I)	1122 (26-I)	1122 (9-I)	1121 (26-I)	1121 (9-I)
Preparation	Whole serum	Supernatant fluid	CO_2 precipitate*	CO_2 precipitate*	CO_2 precipitate*	CO_2 precipitate*
Serum amount *cc.*						
0.01	0†	0	T	T	T	T
0.008	0	0	T	T	T	T
0.006	0	0	T	T	T	T
0.004	0	0	T	T	T	T
0.002	0	0	++++	++++	++++	+++
0.001	0	0	++++	++++	++++	++++
0.0008	0	0	++++	++++	++++	++++
0.0006	T	0	++++	++++	++++	++++
0.0004	T	0	++++	++++	++++	++++
0.0002	T	T	++++	++++	++++	++++
0.0001	++++	++++	T	T	++++	++++
0.00008	++++	++++	0	T	++++	T
0.00006	++++	++++	0	0	+++	T
0.00004	++++	++++	0	0	T	T
0.00002	+++	+++	0	0	T	0
0.00001	T	T	0	0	0	0
0.000008	0	0	0	0	0	0
0.000006	0	0	0	0	0	0
0.000004	0	0	0	0	0	0
0.000002	0	0	0	0	0	0

* Serum heated to 56–57° for ½ hour before precipitation with CO_2.
† Terms as in Table I.

to 1/20 with distilled water has been precipitated with CO_2. If the CO_2 precipitate is redissolved in salt solution, and the salt content of the supernatant fluid restored, comparison shows that the CO_2 precipitate gives a similar reaction but the zone of precipitation is moved toward the stronger concentrations centering about 0.0006 instead of the tenth dilution of this amount. The fluid remaining after the

removal of the CO_2 precipitate may show no reaction whatever. In order to achieve this complete separation it is necessary that the serum be heated to 58°C. for 2 hours before the CO_2 precipitation. If CO_2 precipitation is done on fresh serum or on serum heated for a shorter time, or to 56°C., the separation is at least imperfect.

TABLE III.

Serum	1162	1162	1116	1116
Preparation	Supernatant fluid	CO_2 precipitate*	Supernatant fluid	CO_2 precipitate*
Serum amount *cc.*				
0.01	0†	T	0	T
0.008	0	T	0	T
0.006	0	T	0	T
0.004	0	T	0	T
0.002	0	++++	0	++++
0.001	0	++++	0	++++
0.0008	0	++++	0	++++
0.0006	0	++++	0	++++
0.0004	0	++++	0	++++
0.0002	0	++++	0	++++
0.0001	0	++++	d.T	T
0.00008	0	T	d.T	T
0.00006	0	T	d.T	T
0.00004	0	T	d.T	0
0.00002	d.T	0	d.T	0
0.00001	0	0	d.T	0
0.000008	0	0	0	0
0.000006	0	0	0	0
0.000004	0	0	0	0
0.000002	0	0	0	0
0.000001	0	0	0	0

* Serum heated to 58–60° for 2 hours before CO_2 precipitation.

† Terms as in Table I. d.T = questionable increase in turbidity.

Table II gives the results of serum of Calf 1122, bleeding of January 26, 1925, and compares the whole serum, the supernatant fluid after CO_2 precipitation and the redissolved CO_2 precipitate for this sample. The CO_2 precipitates of one other sample from Calf 1122 and two samples from Calf 1121 are also shown. In this experiment the whole serum, as used in the test and also as preliminary to the CO_2 pre-

cipitation, was heated to 56–57° for ½ hour. It will be noted that the precipitations with the CO_2 precipitates are much alike. Those from Calf 1121 show a somewhat wider range. It is especially interesting that in the case of Serum 1122, the whole serum and the supernatant fluid react alike and yet the precipitate is likewise fully active.

Table III gives the results with the two samples of serum which were heated to 58–60° for 2 hours preliminary to the CO_2 precipitation. In this case the supernatant fluid is without activity while the precipitates are comparable to those shown in Table II.

The animals whose serum was used in the experiments of Table II were both infected with a virulent culture about 1 month before the samples were drawn. They had fever at this time. They had not been immunized.

The samples used in the experiments of Table III were of animals also used in those of Table I, 1162 being normal, 1116 immunized and infected.

The complement fixation reaction must, of course, be carried out with much smaller quantities of the antigen than those which show the precipitation reaction, these being anticomplementary. The fixation value of the sera in question has ranged from a maximum of 0.004 in the case of the immune sera to an immeasurable minimum of less than 0.01 in the case of the normal samples. In certain cases we have added the hemolytic system to the series after precipitation was complete, incubating the complement with the mixtures for varying lengths of time. The results have been so irregular as to be incapable of interpretation.

In one experiment, the tubes showing precipitation were pooled and filtered through paper, the filtrate being tested by complement fixation for the presence of antigen. The test was negative, showing that the specific antigenic substance with which we are dealing is included in the precipitate. The amount of serum involved in the tubes showing precipitation is much less than that required to demonstrate the fixation antibody in any of the serum samples in question and the relation of the precipitate to the immune principles of an active serum remains undetermined.

Finally the sera of a number of other species were tested; horse, sheep, goat, rabbit, guinea pig and fowl were included and all gave

precipitation. The sera were heated to 56°C. for ½ hour before the test. The reaction zones as expressed in serum concentration vary in extent and location but no distinctive character was developed by any. These results are summarized in Table IV.

TABLE IV.

Serum* of.....	Horse	Sheep	Goat	Rabbit	Guinea pig	Fowl
Serum amount cc.						
0.01	0	0	0	0	0	T
0.008	0	0	0	0	0	T
0.006	0	0	0	0	0	T
0.004	0	0	0	0	0	T
0.002	0	0	T	0	T	++++
0.001	T	T	++++	0	T	++++
0.0008	T	++++	++++	0	T	++++
0.0006	++++	++++	++++	0	T	++++
0.0004	++++	++++	++++	T	++++	++++
0.0002	++++	++++	++++	++++	++++	++++
0.0001	++++	++++	++++	++++	++++	T
0.00008	++++	++++	++++	++++	++++	T
0.00006	T	T	++++	++++	++++	T
0.00004	T	++++	++++	++++	T	T
0.00002	T	T	T	T	T	0
0.00001	T	T	0	T	0	0
0.000008	T	0	0	T	0	0
0.000006	0	0	0	0	0	0
0.000004	0	0	0	0	0	0
0.000002	0	0	0	0	0	0

* Sera heated 56–57° for ½ hour.

DISCUSSION.

The experiments present a precipitation reaction between normal serum and an extractive preparation of the tubercle bacillus. The reaction is evidently a very delicate one considering the quantities of material involved, comparing in this respect with rather highly developed immune reactions. The quantities being adjusted on the other hand the phenomenon has the greatest constancy.

The reaction considered as an analog of immune precipitin reactions, which it certainly resembles in its main features, is of

interest in that it presents so wide a pro-zone. The serum dilutions in which activity is evident are quite beyond the range of those it would usually be thought necessary to examine in cases where the more concentrated numbers of the series failed to react, and the discovery of the precipitation was quite accidental.

The results with the fractions of serum after CO_2 precipitation, suggest some direct connection of the serum globulins with the precipitation. Conventional reasoning in consideration of the observation that in the case of serum heated to 56°C. the precipitate and the residual fluid are both active while after heating to 58°C. for a longer time, only the globulin fraction is active, would suggest that a multiplicity of substances might be involved.

The reaction may possibly be another aspect of a result obtained by Wadsworth and Maltaner (13), who found that if crude antigenic preparation from the tubercle bacillus were mixed with 5 volumes per cent of normal horse serum, and precipitated by CO_2, the antigen active in the complement fixation reaction was carried down on the globulin and could be extracted from the precipitate with alcohol.

SUMMARY.

The prolonged extraction of the tubercle bacillus with boiling ethyl alcohol, followed by one or more reprecipitations by chilling the hot alcoholic solution, easily yields a preparation very active as antigen in the complement fixation reaction.

This preparation gives a precipitation reaction with high dilution of the normal blood serum of a number of species.

The precipitation reaction presents as a peculiar feature a very long pro-zone and is further dependent on a preceding heat treatment of the serum for its demonstration.

Occurring as a reaction of normal serum, the reaction is apparently not influenced by immunization sufficient to develop moderate specific complement fixation reactions.

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STUDIES ON IMMUNITY TO PNEUMOCOCCUS MUCOSUS (TYPE III).

I. Antibody Response of Rabbits Immunized with Type III Pneumococcus.

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The absence of demonstrable type specific antibodies in the serum of rabbits immunized with Type III pneumococcus has been the common experience of investigators working with this organism (1–4). Yoshioka (5) obtained type specific agglutinins in the serum of immunized rabbits, but with irregularity. He attributed his positive results to the use of one strain of Type III, since, with two other strains, no specific antibody production was elicited. Immunization of sheep and horses with Type III pneumococcus has resulted in the development of type specific agglutinins in the sera of these animals (6), but the titre has been low and passive protection slight.

This experience with *Pneumococcus mucosus* is but a confirmation of the earlier observations (7) that the serum of animals immunized with certain of the encapsulated organisms fails to show agglutinins. This failure to demonstrate antibodies in serum under these conditions has been usually attributed to the inagglutinability of organisms possessing a mucoid capsule or to the absence of agglutinins in the test serum. Porges (8) found that if encapsulated Friedländer bacilli were first subjected to a process of decapsulation, they then became agglutinable in immune serum. Hanes (1) reported that Type III pneumococci were agglutinated in their homologous immune serum following decapsulation of the organisms by means of the Porges method.

In the course of a series of investigations on immunity to Type III pneumococcus, the response of rabbits to immunization with this organism was investigated.

EXPERIMENTAL.

Antigen.—Three different strains of Type III pneumococcus were employed. These strains were derived from the blood of patients suffering from lobar pneumonia and possessed all the characteristics of Type III. They were Gram-positive diplococci; they showed capsule formation when suitably stained; they grew on blood agar plates with the production of large mucoid colonies; they were bile-soluble; they agglutinated promptly in Type III antipneumococcus horse serum; they were virulent for mice in 0.0000001 cc. doses. Both living organisms and vaccine were used for injection. A 16 hour plain broth culture was used for the living organisms. The vaccine was made by centrifuging such a culture, resuspending the organisms in physiological salt solution, and heating at 56° for half an hour.

Methods of Immunization.—Three methods of immunization were employed. (1) The intravenous injection of vaccine according to the method described by Cole and Moore (9). This method consists in alternating for 6 weeks a week of daily injections of 1 cc. of vaccine with a week of rest. (2) Following a week of intravenous vaccine injections of 1 cc. each day, living organisms were injected at 3 to 4 day intervals in increasing amounts from 0.1 to 10 cc. over a period of 4 weeks. (3) Living organisms were injected intravenously from the beginning of immunization; 1 cc. was given daily for 6 days followed by increasing doses from 3 to 10 cc. at 3 to 4 day intervals over a period of 4 weeks.

All animals were bled 10 to 12 days after the last immunizing dose.

Agglutinin Reaction.—0.5 cc. of a saline suspension of heat-killed organisms was added to an equal volume of the serum dilutions. In the tests with type specific pneumococci, a positive reaction, if present, usually appeared immediately and the final reading was made after the tubes had remained 2 hours in the water bath at 37°C. When decapsulated Type III pneumococci or R strains of pneumococci were used, the reading was made after the tubes had remained 2 hours in the water bath at 37°C. and overnight in the ice box.

Precipitin Reaction. Nucleoprotein.—The nucleoprotein solutions of Pnuemococcus used as precipitinogen in the tests were made by the method described by Avery and Morgan (10). They contained approximately 3 mg. of N per cc. 0.2 cc. of the test serum diluted to 0.5 cc. by the addition of physiological salt solution was added to 0.5 cc. of the nucleoprotein solution. Readings were made after 2 hours in the water bath at 37°C. and overnight in the ice box.

Soluble Specific Substance.—This substance, representing the N-free carbohydrate derived in a purified state from Type III pneumococci according to the method described by Heidelberger and Avery (11), was used in dilutions of 1–10,000, 1–20,000, 1–40,000, and 1–100,000. Dilutions of this degree were employed in order to escape the inhibition zone experienced (12) when higher concentrations are tested. 0.2 cc. of serum diluted to 0.5 cc. by the addition of physiological salt solution was added to 0.5 cc. of the different dilutions of the soluble specific substance. The precipitin reaction is usually immediate; however, the final reading was made after 2 hours in the water bath at 37°C.

Passive Protection Tests.—These tests were carried out in mice by the simultaneous injection intraperitoneally of 0.2 cc. of serum and culture dilution, according to the method previously described (13).

The results obtained in the experiments to be reported involve the activities of two antigen-antibody systems. In order to avoid confusion as to nomenclature, certain terms used throughout the body of the report will be defined. It has been shown by Avery and Heidelberger (14), that pneumococci are capable of stimulating the production of two distinct antibodies, depending in a large measure on the nature of the bacterial substances used as antigens. An intact pneumococcal cell possessing the soluble specific substance, when employed antigenically, stimulates the production of antibodies which are reactive with the homologous soluble specific substance. If the soluble specific substance is present in the cells used in the agglutination test, type specific agglutination occurs; if the soluble specific substance is present in solution, precipitation of this substance occurs. The antibody, which is capable of agglutinating the type specific Pneumococcus and of precipitating the type specific soluble substance will be designated as *anti-S*. The evidence of all previous work indicates that the efficacy of serum in the passive protection of mice against pneumococcus infection depends on the presence in the serum of anti-S.

The second antigen-antibody system involves the nucleoprotein fraction of the cell and the antibody response elicited by it. Pneumococcus nucleoprotein, in contrast to the soluble specific substances, is without type specificity. The degraded R strains of pneumococci also are without type specificity. Therefore the use, antigenically, of either nucleoprotein substance or R strains results in the production of non-type specific antibodies, which precipitate the nucleoprotein derived from any Pneumococcus and agglutinate all R strains. These antibodies will be designated as *anti-P*.

I. Agglutinins.

Absence of Agglutinins for the Encapsulated Type III Pneumococcus in the Sera of Animals Immunized with Type III Pneumococcus.

Twenty-eight rabbits were used in these immunization experiments. Twenty received vaccine alone; six received vaccine and living organ-

isms; two received living organisms alone. Their sera were then tested for the presence of agglutinins against the encapsulated Type III pneumococcus. Out of the twenty-eight rabbits, the sera of twenty-four failed to show type specific antibodies (anti-S). In the four positive sera type specific antibodies (anti-S) were present in low titre and in only one was the agglutinin reaction positive in a dilution of 1 to 20 (Table I). Such results are confirmatory of the experience of others in working with Type III and are in striking contrast to the results obtained when rabbits are immunized with Pneumococcus Type I or Type II, whereby type specific antibodies are readily demonstrable in the immune sera.

Presence of Agglutinins for R Strains of Pneumococcus in the Sera of Rabbits Immunized with Type III Pneumococcus.

In demonstrating the twofold antigenicity of pneumococcus substances, Avery and Heidelberger (14) showed that when intact pneumococcal cells are used as antigen the resultant antibodies consist chiefly of type specific agglutinins (anti-S) for the homologous organism; when ruptured cells or solutions of pneumococci are used as antigen the antibody response is predominantly of the anti-P character. Since the sera of rabbits immunized with Type III pneumococcus fail, in most instances, to show anti-S antibodies, and in view of the dual antigenic nature of pneumococci, these immune sera were tested for the presence of anti-P antibodies. Anti-P antibodies are reactive against the degraded R strains of Pneumococcus. As has been brought out by Reimann (15, 16) R strains are avirulent, non-encapsulated, and non-type specific. They may be derived from any of the fixed types of Pneumococcus and are agglutinated by any anti-P serum. Therefore, R strains of pneumococci are adequate in testing for the presence of anti-P antibodies. The results recorded in Table I were obtained by testing the sera of rabbits, immunized with Type III pneumococcus, for the presence of anti-P antibodies, as evidenced by the agglutination of an R strain. The sera of twenty-seven of twenty-eight rabbits possessed anti-P antibodies.

The results of this experiment indicate that rabbits immunized with Type III pneumococcus—even though this organism is one of the fixed types—react in the majority of instances by the production, not

of the type specific anti-S antibody, but of the anti-P antibody. The immunological response is identical with that obtained following immunization with R strains or solutions of pneumococci. The inference is, then, that Type III pneumococci are so altered, following introduction into the animal body, that the type specific antigen is made ineffectual and the nucleoprotein is liberated to stimulate the production of anti-P antibodies.

Agglutination of Decapsulated Pneumococci in the Sera of Rabbits Immunized with: (1) Type III Pneumococcus, (2) R Strains of Pneumococcus, (3) Nucleoprotein Derived from Pneumococcus.

Hanes (1), as previously stated, showed that Type III pneumococci, deprived of their capsule by means of the Porges (8) method, were agglutinated in the sera of rabbits immunized with Type III pneumococci; whereas, when the encapsulated organisms were tested in the same immune sera, no agglutination occurred. Both Porges and Hanes infer that the inagglutinability of encapsulated organisms is due, not to the absence of agglutinins in the test serum, but to the presence of the capsule around the organism. As proof of this they show that removal of the capsule is followed by agglutination.

Since it has been shown in Table I that rabbits immunized with Type III pneumococcus possess anti-P antibodies and, since the chief characteristic of anti-P antibodies is the agglutination of non-encapsulated pneumococci, an explanation of the results of Hanes is made possible. R strains of pneumococci agglutinated by Type III immune rabbit serum (Table I) are organisms which have been deprived of their capsule by cultural methods. The Type III organisms which Hanes found agglutinable were deprived of their capsule by a chemical procedure. The agglutination of both of these non-encapsulated organisms is identical (Table I) in Type III immune rabbit serum. Their immunological identity is further brought out by testing the agglutination of chemically decapsulated organisms in the sera of rabbits immunized with R strains and with pneumococcus nucleoprotein. The results (Table II) show that any anti-P serum is capable of agglutinating the chemically decapsulated Type III pneumococcus and R strains equally well. In reports on Friedländer bacilli Julianelle (17) obtained similar results; namely, that decapsulated and R

TABLE I.

Agglutination of Encapsulated Type III Pneumococcus, Decapsulated Type III Pneumococcus, and of an R Strain of Pneumococcus in the Sera of Rabbits Immunized with Type III Pneumococcus.

Rabbit No.	Agglutinins for Pneumococcus III encapsulated					Agglutinins for Pneumococcus III decapsulated					Agglutinins for R_3 strain of pneumococcus					
	Serum dilutions					Serum dilutions					Serum dilutions					
	1–2	1–10	1–20	1–40	1–80	1–10	1–20	1–40	1–80	1–160	1–2	1–10	1–20	1–40	1–80	1–160
1	−	−	−	−	−	++++	+++	+	±	−	++++	++++	+++	++	±	−
2	−	−	−	−	−	++++	++++	++	+	+	++++	++++	++	+	−	−
3	−	−	−	−	−	++++	++	+	−	−	++++	+++	++	+	−	−
4	−	−	−	−	−	++++	+++	+	±	−	++++	++++	++	+	−	−
5	−	−	−	−	−	++++	++++	+++	+	±	++++	++++	+++	++	+	±
6	−	−	−	−	−	++++	+++	+	±	−	++++	+++	+	±	−	−
7	−	−	−	−	−						+++	++	+	−	−	−
8	−	−	−	−	−						++++	++++	+++	++	+	±
9	−	−	−	−	−						++++	+++	++	+	−	−
10	−	−	−	−	−						++++	+++	++	+	±	−
11	−	−	−	−	−						+++	++	±	+	−	−
12	−	−	−	−	−						++++	+++	++	++	±	−
13	−	−	−	−	−						++++	+++	++	+	−	−
14	−	−	−	−	−						++++	++++	+++	++	+	−
15	××××	××	×	×	−						+	±	−	−	−	−
16	×××	×	−	−	−						++	++	+	−	−	−
17	××	×	−	−	−						++++	+++	+	±	−	−
18	−	−	−	−	−						++++	++++	++	++	±	−
19	−	−	−	−	−						++++	++++	+++	+	−	−
20	−	−	−	−	−						±	−	−	−	−	−
21	−	−	−	−	−						+++	+	±	−	−	−
22	−	−	−	−	−						++++	+++	+	±	−	−

23	−	−	−	−	−						++++	++++	++	+	±	−
24	−	−	−	−	−						++++	++++	+++	++	+	±
25	−	−	−	−	−						++++	+++	++	+	−	−
26	−	−	−	−	−						++++	++++	++	+	±	−
27	××××	××	×	−	−						++++	+++	+	±	−	−
28											++++	++++	++	+	±	−

Controls: 0.5 cc. organisms + 0.5 cc. of salt solution: diffuse. 0.5 cc. organisms + 0.5 cc. of normal rabbit serum: negative.

×××× indicates characteristic compact disc of type specific agglutination with clear supernatant fluid. ××× indicates compact disc with faintly cloudy supernatant fluid. ×× indicates disc which can be partially broken up with cloudy supernatant fluid. × indicates coarse granulation. $\underline{\times}$ indicates doubtful granulation. − indicates negative.

++++ indicates complete agglutination with clear supernatant fluid. +++ indicates less coarse agglutination with faintly cloudy supernatant fluid. ++ indicates agglutination with cloudy supernatant fluid. + indicates some granulation both sedimented and diffuse. ± indicates some sedimentation easily made diffuse. − indicates negative.

TABLE II.

Agglutination of Decapsulated Type III Pneumococci and of R Pneumococci in the Sera of Rabbits Immunized with Two Different R Strains of Pneumococcus and with Pneumococcus Nucleoprotein.

		Decapsulated Type III						R_2 pneumococci					
Rabbit No.	Immunized with	1–10	1–20	1–40	1–80	1–160	1–320	1–10	1–20	1–40	1–80	1–160	1–320
29	R_1 strain	++++	++++	+++	+	±	−	++++	+++	++	+	±	−
30	R_2 strain	++++	+++	+	±	−	−	++++	+++	+	−	−	−
31	Nucleo-protein	++++	+++	++	+	±	−	++++	+++	++	+	−	−

Controls: 0.5 cc. of organisms + 0.5 cc. of salt solution: diffuse. 0.5 cc. of organisms + 0.5 cc. of normal rabbit serum: some sedimentation which was made diffuse by slight tapping.

++++ indicates complete agglutination with clear supernatant fluid. +++ indicates less coarse agglutination with faintly cloudy supernatant fluid. ++ indicates agglutination with cloudy supernatant fluid. + indicates some granulation both sedimented and diffuse. ± indicates some sedimentation easily made diffuse. − indicates negative.

strains of Friedländer bacilli were identical in their agglutination reactivity in anti-P (Friedländer) serum.

II. Precipitins.

It was shown by Reimann (16) that sera which contained agglutinins (anti-P) for R strains of Pneumococcus also contained precipitins for pneumococcus nucleoprotein solutions. Therefore, since the sera of the rabbits immunized with Type III pneumococcus possessed anti-P agglutinins, they were tested for the presence of precipitins for pneumococcus nucleoprotein (Table III).

A comparison of Table III with Table I will show that all the sera which contained anti-P agglutinins also possessed anti-P precipitins. Since the nucleoprotein in this test was obtained from a Group IV pneumococcus, the possibility of a type specific reaction is excluded and the experiment further identifies the antibody present in the Type III immune sera as of an antiprotein character.

The sera of the rabbits immunized to Type III were also tested for the presence of precipitins against Type III specific soluble substance (Table III). As has been shown by Avery and Heidelberger (14), the type specificity of pneumococci depends upon the presence of this substance. They have obtained it in a highly purified state from each of the three fixed types of Pneumococcus. Immune sera which possess type specific agglutinins (anti-S) for pneumococci of a fixed type also possess precipitins for the soluble specific substance derived from that type. The results obtained with Type III immune rabbit sera used in these experiments show the concomitant occurrence of Type III specific agglutinins (anti-S) and precipitins for the Type III soluble specific substance. On the other hand, those sera which failed to show type specific agglutinins (anti-S) also failed to precipitate the soluble specific substance. This is further evidence that the lack of agglutination of the encapsulated Type III cell in homologous immune rabbit serum is due to the actual absence of the anti-S antibody in demonstrable quantity since such factors as were supposed to render the encapsulated organism inagglutinable are not present in the precipitin test.

TABLE III.

Precipitation of the Soluble Specific Substance of Type III Pneumococcus and of a Solution of Pneumococcus Nucleoprotein in the Sera of Rabbits Immunized with Type III Pneumococcus.

Rabbit No.	Precipitins for soluble specific substance of Type III pneumococcus				Precipitins for solution of pneumococcus nucleoprotein
	Dilutions of soluble specific substance				Dilution of protein
	1–10,000	1–20,000	1–40,000	1–100,000	1–300
1	–	–	–	–	+++
2	–	–	–	–	++
3	–	–	–	–	++
4	–	–	–	–	++
5	–	–	–	–	+++
6	–	–	–	–	++
7	–	–	–	–	++
8	–	–	–	–	+++
9	–	–	–	–	++
10	–	–	–	–	++
11	–	–	–	–	++
12	–	–	–	–	++
13	–	–	–	–	++
14	–	–	–	–	++
15	××××	××	×	×	+
16	××××	×	×	–	±
17	×××	×	–	–	++
18	–	–	–	–	++
19	–	–	–	–	++
20	–	–	–	–	–
21	–	–	–	–	+
22	–	–	–	–	++
23	–	–	–	–	++
24	–	–	–	–	+++
25	–	–	–	–	++
26	–	–	–	–	++
27	××××	×	×	–	++
28	–	–	–	–	+++

Controls: 0.5 cc. of various dilutions of soluble specific substance + 0.5 cc. salt solution: clear. 0.5 cc. of various dilutions of soluble specific substance +0.2 cc. of normal rabbit serum + 0.3 cc. of salt solution: clear. 0.5 cc. of pneumococcus nucleoprotein solution + 0.5 cc. of salt solution: slightly hazy

III. Passive Protection in Mice.

The sera of the twenty-four rabbits immunized with Type III pneumococcus were tested for their ability to protect mice against infection with Type III pneumococcus. The dependence of passive protection on the presence of type specific antibodies (anti-S) in the test serum is well known to investigators working in experimental pneumococcus immunity. Therefore, it is to be expected that those sera containing anti-S afford some protection and that those without it fail. The results recorded in Table IV show that protection parallels the presence of anti-S and not anti-P. Of the twenty-four sera tested, the four possessing anti-S showed protection; of the twenty possessing no demonstrable anti-S, seventeen afforded no protection, and three afforded very slight protection. The explanation of the results obtained with the latter three sera probably rests on the fact that these sera contained sufficient type specific antibodies (anti-S) to afford some protection but not sufficient to be demonstrable by test-tube agglutination. That the mouse protection test is a more delicate test for the presence of anti-S than test-tube agglutination may be brought out by the use of Type I antipneumococcus serum. This serum may be diluted to a point where no test-tube agglutination is demonstrable and yet such a dilution of serum affords some protection. A further report will be made later on the results obtained in passive protection following the use of the sera of rabbits immunized with Type III pneumococci.

An analysis of the antibodies present in the sera of twenty-eight

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0.5 cc. of pneumococcus nucleoprotein solution + 0.2 cc. of normal rabbit serum + 0.3 cc. of salt solution: slightly hazy.

×××× indicates characteristic compact disc of type specific precipitin reaction with clear supernatant fluid. ××× indicates compact disc with faintly cloudy supernatant fluid. ×× indicates smaller disc at bottom of tube with cloudy supernatant fluid. × indicates cloudy fluid. × indicates very faintly cloudy fluid. − indicates negative.

+++ indicates precipitate at bottom of tube with faintly cloudy supernatant fluid. ++ indicates slight precipitate with cloudy supernatant fluid. + indicates cloudy fluid. ± indicates very faintly cloudy fluid. − indicates negative.

rabbits immunized with Type III pneumococcus may be summarized as follows:

Agglutinins.—Twenty-four failed to show type specific agglutinins (anti-S). Four possessed type specific agglutinins (anti-S) in low titre. Twenty-seven possessed anti-P agglutinins in appreciable titre. One failed to show evidence of any antibody response.

TABLE IV.

Summary of Passive Protection in Mice by the Use of Sera of Rabbits Immunized with Type III Pneumococcus.

	Demonstrable anti-S antibodies		Demonstrable anti-P antibodies		Rabbit No.	Passive protection in mice against Type III pneumococcus infection. Dose of culture			
	Agglutinins	Precipitins	Agglutinins	Precipitins		0.001 cc.	0.0001 cc.	0.00001 cc.	0.000001 cc.
Sera of 17 rabbits	Absent	Absent	Present	Present		D.	D.	D.	D.
Sera of 3 rabbits	Absent	Absent	Present	Present	6	D.	D.	D.	S.
					11	D.	D.	D.	S.
					12	D.	D.	S.	S.
Sera of 4 rabbits	Present	Present	Present	Present	15	S.	S.	S.	S.
					16	S.	S.	S.	S.
					17	D.	S.	S.	S.
					27	D.	S.	S.	S.

D. indicates death of animal.
S. indicates survival of animal.

Precipitins.—The presence of precipitins for Type III soluble specific substance paralleled the presence of type specific agglutinins. The presence of precipitins for pneumococcus nucleoprotein paralleled the presence of anti-P agglutinins.

Passive Protection in Mice.—Four sera possessing demonstrable anti-S antibodies afforded some protection against Type III infection.

Of twenty sera without demonstrable anti-S, seventeen failed to confer any protection; three afforded minimal protection.

DISCUSSION.

The common experience that Type III pneumococci, antigenically employed, fail to stimulate the production of type specific agglutinins (anti-S) has been encountered in a great majority (85.8 per cent) of the rabbits used in the experiments here reported. It is a striking fact that such a cell, possessing a large amount of soluble specific substance, and highly virulent for mice, acts so feebly in stimulating type specific antibodies (anti-S); whereas Type I and Type II pneumococci, possessing the same qualities, are so effective in producing homologous type specific antibodies. However, the presence of agglutinins for R pneumococci and precipitins for pneumococcus nucleoprotein (anti-P antibodies) is evidence that Type III pneumococci are not without an antigenic component. The results of these experiments reveal the fact that the antibody response of rabbits to immunization with the encapsulated Type III pneumococcus is identical with the antibody response obtained by immunization with solutions of pneumococci. In both instances the production of anti-P antibodies has been stimulated. Such a result could be accomplished only by *in vivo* disruption of the pneumococcal cells. The inference, then, may be drawn that normal rabbits possess a mechanism whereby Type III pneumococci, following intravenous injection, are disintegrated in such a manner that the part of Type III antigenic complex, which stimulates type specific agglutinins (anti-S), is destroyed. The nucleoprotein fraction of the cell, however, remains capable of stimulating anti-P antibodies.

The identification of the antibody present in Type III immune rabbit serum, as being anti-P, is explanatory of Hanes' results with decapsulated Type III pneumococci. By decapsulation, Type III pneumococci are reduced to an R form, and are agglutinated by anti-P antibodies. The failure of encapsulated Type III to agglutinate is due to the actual absence of anti-S antibodies.

CONCLUSIONS.

1. Type III pneumococci fail in the majority of instances to stimulate the production of anti-S antibodies. (Type specific agglutinins, type specific precipitins, and antibodies affording type specific protection in mice.)

2. Type III pneumococci are effective in the stimulation of the production of anti-P antibodies (agglutinins for R strains of pneumococci and precipitins for pneumococcus nucleoprotein). These antibodies are ineffectual in the passive protection of mice.

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INFLUENCE OF LIGHT ON THE GROWTH AND MALIG NANCY OF A TRANSPLANTABLE NEOPLASM OF THE RABBIT.

Second Paper.

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The experiments reported in the present paper are a continuation of previous work dealing with the effect of light on the growth and malignancy of a transplantable epithelial neoplasm of the rabbit (1) and are a part of a general study on the relationship of external conditions to the physical constitution of normal rabbits and to the reaction of rabbits to certain experimental diseases.

The belief that there is a relationship between sunlight and the manifestations of this particular tumor arose, first, from the fact that a characteristic feature of the disease is the seasonal variability of its behavior and, second, that a correlation could be drawn between the prevailing level of sunshine and changes in the curve of sunlight, on the one hand, and variations in malignancy of the tumor process, on the other (2). In order to test this belief, experiments were carried out in which conditions of light could be controlled. The results of the first experiment (1) showed that, as compared with rabbits living under ordinary indoor conditions of diffused sunlight, a slightly lower plane of malignancy obtained in a group of rabbits kept in constant darkness but that a very low level prevailed in the group exposed to constant light. The particular type of lighting (Mazda lamps and mercury arcs in crown glass) employed in this experiment, however, precluded its use except during the colder months, so that a different arrangement was necessary to permit experiments at other seasons of the year.

In the present paper, 4 experiments are reported in which were

studied the effect of constant illumination with Cooper Hewitt mercury arcs in crown glass and the effect of constant darkness upon the general course and manifestations of the malignant disease.

Methods and Material.

The experiments were carried out from October 1, 1925, to April 12, 1926. 3 animal rooms with similar environmental conditions other than those of lighting were employed. These rooms which will be referred to as the light, the dark and the control or unaltered room, respectively, have already been described in connection with the 1st experiment (1). It is sufficient in the present report merely to call attention to the lighting arrangement of the light room which was the only feature in the equipment of the 3 rooms that was changed.

A *constant* source of light was furnished by 13 Cooper Hewitt low pressure mercury arcs, Type P, in crown glass arranged in 3 superimposed rows on an iron frame placed in the center of the room. All other light was excluded. The rabbit cages were placed in racks on either side of and parallel with the mercury arcs. The intensity of light reaching the cages was not entirely uniform, those in the central portions of the racks receiving more light than those at the top and bottom or at the ends. The average intensity was 200 foot candles.

The spectrogram of the mercury arcs in crown glass shows that the range of light rays is from 3022 to 5790 Ångström units with the majority falling in the 3650 and 5790 portion of the spectrum.

The temperature of the 3 rooms was satisfactorily maintained at 70° to 75°F. and the humidity of each varied with that of the outside air.

The rabbits employed were males, chosen from a selected stock and were matched as to breed and type. The majority were approximately 8 to 12 months old. Each animal was separately caged and all were fed a similar diet of hay, oats and cabbage.

The experiments were all conducted in the same manner. Comparable groups of rabbits were placed in each room at the same time where they remained for the duration of the experiment. After varying intervals, each group was inoculated with the tumor. All inoculations were made in one testicle with 0.3 cc. of an emulsion of an actively growing primary tumor. This neoplasm is considered to be of epithelial origin (3).

Frequent examinations of the rabbits were made, special attention being given to the general physical condition of the animals, the character of the primary tumor and the detection of metastases recognizable during life. In addition, the rabbits were weighed at regular intervals. Certain animals which developed signs and symptoms of impending death were killed by an injection of air into the marginal ear vein. The experiments were terminated 2 months after inoculation at which time all surviving animals were killed. A complete postmortem examination of each rabbit was made with special reference to the condition of the primary tumor,

the incidence of metastatic tumors and the number, distribution, size and state of these growths.

The observations thus obtained have been used as a basis for evaluating the effect of the particular light condition upon the general level of malignancy. The results are considered from a group standpoint in order to minimize the factor of individual animal variation.

The method used in analyzing the data on metastatic foci obtained at post-mortem examination requires some explanation. First, the sites or foci of secondary tumors, not their actual number, are counted and hence the expressions "foci of metastases," "metastatic rate" or "distribution of metastases." On the other hand, the number of metastases detected during life refers to the actual number found.

Second, in order to classify the character of the disease in such general terms as high, moderate and low levels of malignancy, the metastatic foci have been grouped in the following divisions:

I. Suprarenals and eyes	4	possible foci
II. Extensions and implantations to the retroperitoneal and mediastinal tissues, omentum, mesentery and parietal peritoneum	19	" "
III. Lungs and pleura, liver, kidneys and pancreas	5	" "
IV. Skin and subcutaneous tissue, superficial lymph nodes, muscles, heart and pericardium, bones and bone marrow, glands of internal secretion with the exception of the suprarenals, the spleen and the central nervous system	30	" "

The reasons for this classification have been stated in the paper describing the results of the first experiment (1). Suffice it to say here that in instances of low malignancy, metastases may be found only in the suprarenals and eyes, while in cases of high malignancy with death in 3 to 5 weeks after inoculation there may be a widespread distribution of tumor to many tissues and organs including the skin, muscles, bones and glands of internal secretion which are practically never involved in instances of low malignancy. An occasional exception to this rule is met with in certain cases of tumors in the cranial sinuses or facial bones which may be the only metastases found.

In the third place, the distribution of metastases has been considered upon a percentage basis of the possible sites of such growths as determined by the actual location of metastases in the first 20 generations of the tumor (4). The percentage involvement for each rabbit was first obtained and these values were then combined on a group basis to form what have been called "summation" values of metastatic foci.

Results.

The results of the 4 experiments consisting of clinical and post-mortem observations are presented in 4 tables and 2 charts, the latter being graphic representations of the distribution of metastatic foci. (Tables I, II, III and IV; Text-figs. 1 and 2.) The experiments are arranged according to the length of exposure to constant illumination or constant darkness prior to inoculation, that is, Nos. I and II, 4 weeks; No. III, 5 weeks and No. IV, 16 weeks. Since the duration of observation subsequent to inoculation was in each case 2 months, the entire period of exposure to these conditions was as follows: Nos. I and II, 12 weeks; No. III, 13 weeks and No. IV, 24 weeks. The results of each experiment are presented in the order of the control, the dark and the light groups, respectively.

Analysis and Discussion of Results.

The purpose of these experiments was to determine whether the constant exposure of rabbits to light supplied by mercury arcs in crown glass and to continuous darkness influenced the general course and character of a malignant tumor. Recognizing the fact that an adequate interpretation of results obtained in experiments of this nature implies a consideration of the tumor process in as comprehensive a way as possible, the present analysis has included all features of the disease which are believed to be significant. In addition, the analysis has been made on a group basis in order to avoid individual animal variation, and the results obtained under the experimental light conditions have been compared, in each instance, with those of appropriate control groups. It will aid to a clearer understanding of the evaluation of these comparisons if a brief description of the disease in the 4 control groups be given first.

General Character of the Disease.—The most striking feature of the tumor process in the control groups was the relatively low level of malignancy which prevailed in all 4 experiments. There was a considerable variation in disease severity among the groups but in none of them was the average plane of malignancy high.

As far as general procedure was concerned, the experiments differed in only 2 particulars, that is, in the time of year at which

they were carried out and second, in the length of exposure to constant illumination and to constant darkness before inoculation which, of course, does not concern the control animals. The dates of inoculation were as follows:

Experiment I, October 27, 1925.
" III, November 2, 1925.
" IV, January 29, 1926.
" II, February 11, 1926.

The most pronounced disease developed in Experiments I and III but the general plane of malignancy was low as compared with many series observed in previous years. An even less severe disease occurred in the 2nd and 4th experiments, and in the case of No. II, the condition was extremely mild. The generally low level of malignancy as well as the group variations in disease severity are well illustrated by the differences in the mortality rates and in the incidence of metastases determined at postmortem examination as shown by the following data:

	No. of rabbits	Mortality rate	Incidence of metastases
		per cent	*per cent*
Experiment I	11	36.4	81.9
" III	11	27.3	63.6
" IV	5	20.0	40.0
" II	10	0	30.0
Weighted average	37	21.6	56.8

Similar variations in metastatic incidence and in the death rate were noted in a study of the first 20 generations of the tumor (5) but there were certain peculiarities of metastatic distribution in the present experiments, which were not wholly characteristic of the disease as previously observed. While this phase of the subject cannot at present be discussed in detail, its occurrence should be noted and its most pronounced feature, namely, metastases to the bones, briefly described because of its bearing upon the experimental results obtained.

As compared with the first 20 generations of the tumor, the incidence and distribution of bone metastases were as follows:

	First 20 generations, tumor	Controls of these experiments
Total No. of rabbits	191	37
Incidence of bone metastases	15 or 7.9 per cent	6 or 16.2 per cent
Proportion of bone metastases to total foci	13.2 per cent	11.6 per cent
Distribution of bone lesions		
Facial bones	12	16
Calvaria	6	0
Thorax	4	1
Posterior extremities	4	2
Spinal column	3	1
Anterior extremities	1	0
Total bone lesions	30	20

Although the disparity in the numbers of animals in the 2 series and the mixed nature of the material comprising the first 20 generations does not strictly permit a comparison, still there are 2 particulars in which the control rabbits of these experiments differed from the larger group. In the first place, there was a much higher incidence of bone lesions—16.2 as contrasted with 7.9 per cent and second, an increase in the proportion of metastases to the facial as compared with other bones (80.0 per cent as contrasted with 40.0 per cent). The ratio of bone metastases to the total number of secondary growths, however, was practically the same in both series. From the standpoint of animal incidence, the bone lesions of the large series were almost equally divided between cases of fulminating malignancy and animals with relatively malignant but slowly progressive tumors. Among the present controls, bone lesions occurred only in cases of comparatively pronounced malignancy. There were no instances in the controls of the 2nd type of malignancy, which is another point in which the character of the disease in these experiments differed from that previously observed. The tumor process of the control rabbits fell, on the whole, into 2 classes, in 1 of which malignancy was fairly well marked while in the other, the disease was very mild as evidenced by the absence of, or the few numbers of, metastases found.

The character of the disease in the groups exposed to constant illumination and to constant darkness did not entirely conform in its general picture to that of the controls, and these differences will be pointed out in the following analysis of the results obtained in these groups.

TABLE I.

Analysis of Clinical Observations.

Experiment No.	Length of exposure before inoculation	Group	No. of rabbits	Primary tumors	Metastases			Mortality		
					Incidence		Total No.	No.	Rate	Time after inoculation
					No.	Per cent				
	wks.								*per cent*	*wks.*
I	4	C.	11	All +	5	45.5	13	4	36.4	4, 5½, 6½*, 7½
		D.	12	All +	4	33.3	7	3	25.0	5, 6, 7½
		L.	12	All +	3	25.0	8	1	8.3	3
II	4	C.	10	4 +	0	——	0	0	——	
		D.	9	2 +	2	22.2	2	1	11.1	7
		L.	10	0 +	0	——	0	0	——	
III	5	C.	11	All +	3	27.3	4	3	27.3	4, 5, 5½
		D.	12	All +	2	16.7	4	2	16.6	4, 6
		L.	10	All +	4	40.0	9	3	30.0	4½, 5, 5½
IV	16	C.	5	2 +	2	40.0	10	1	20.0	6½
		D.	5	2 +	2	40.0	4	1	20.0	6½
		L.	4	2 +	0	——	0	0	——	

C. = controls; D. = groups exposed to constant darkness; L. = groups exposed to constant illumination.

* Complicating empyema.

Clinical Observations.—Under this heading are discussed the primary tumor, the development of metastases recognizable during life and the general physical condition of the animals.

A primary tumor developed in all animals of Experiments I and III (Table I). In the others, however, there were a number of instances as shown below in which no definite primary growth occurred.

	Experiment IV	Experiment II
	per cent	*per cent*
Controls	60.0	60.0
Darks	60.0	77.8
Lights	50.0	100.0

Although the intratesticular route of inoculation has yielded an almost uniform incidence of primary tumors, there have been instances in which none developed. An error in technic with the inoculum deposited in the scrotal sac may have been responsible for some of these failures, but in 2 series inoculated in the fall of 1922, the number of negative results were comparable to those of the present experiments (4). In addition, it may be noted that as far as Experiment IV was concerned, the rabbits had been in the laboratory 6 months prior to inoculation and 4 months under the conditions of the experiment—a period probably sufficient for the establishment of a relatively stable state of the organism. It would appear, and this point will later be taken up in greater detail, that the factors of stability or changeability as applied to external conditions are of prime importance in influencing tumor malignancy, the first manifestation of which is the growth capacity of the transplant.

It was also observed in many rabbits kept in the light room that the testicles were reduced in size and became firmer, conditions which would not necessarily be conducive either to initial or to continued tissue growth. Perhaps this factor in connection with the low degree of malignancy prevailing at the time was largely responsible for the completely negative results as regards the primary tumor in the light group of Experiment II.

The non-development of a primary tumor, however, was not necessarily followed by an absence of growths in other parts of the body as shown by the following data compiled from Experiment II:

	Total No. of rabbits	Negative primary results	Incidence of metastases with no primary tumors
Controls	10	6	1 or 16.7 per cent
Darks	9	7	2 " 28.6 " "
Lights	10	10	1 " 10.0 " "

The character of growth of the primary tumor differed under the various light conditions of the experiments. On the whole, the largest tumors developed in the controls, and in general these groups showed a definite tendency towards a longer persistence of active primary growth as compared with an earlier regression in the tumors of the rabbits from the dark and light rooms. The most irregular type of growth occurred in the rabbits kept in the dark room. During the 1st week after inoculation, little difference was noted between the primary tumors of these rabbits and those of the controls, but in the 2nd and 3rd weeks, the resemblance ceased. It then became difficult to determine from clinical observation whether certain tumors in the dark series were growing. Eventually, some tumors grew more rapidly than those in the controls, but in the majority of instances the speed and degree of growth were less marked.

In the groups kept in the light room, on the other hand, the initial development of the primary tumor was definitely slower and less pronounced than in the controls. The majority of the tumors in these groups did not attain the size of those in the controls and in addition, regression took place earlier and was somewhat more rapid. There were certain instances, however, of very actively growing and extensive primary tumors, but they were not as numerous as in either the dark or the control groups.

The condition of the primary tumors at postmortem examination was, on the whole, in harmony with the general character of the disease at the time this was done, irrespective of the group to which the animal belonged. Thus, in rabbits dying within 4 to 6 weeks after inoculation and in which metastases were found to be more or less widely distributed, the primary tumors showed variable amounts of living tissue. On the other hand, the growths in the animals living to the end of the observation period were in the majority of instances either healed or largely necrotic and heavily encapsulated.

The incidence of metastases recognizable during life among control groups varied in the different experiments from 0 to 45.5 per cent (Table I). While there was only 1 control group (Experiment II) in which no secondary tumors were detected, there were 2 such groups from the light room (Experiments II and IV) and the incidence in the others was 25.0 and 40.0 per cent. There was less variation

among the groups kept in the dark room, the values being 16.7 to 40.0 per cent. If the experiments are combined, the incidence of "clinical" metastases was:

	Total No. of rabbits	No. of rabbits with metastases	Rate
			per cent
Controls	37	10	27.07
Darks	38	10	26.31
Lights	36	7	19.44

The numbers of secondary tumors detected during life were—Controls 27, Darks 17, Lights 17. There was 1 rabbit from the light room (Experiment III) in which a malignant disease developed and the numerous growths in superficial parts of the body of this rabbit considerably augment the total number of clinical metastases of the light groups. On the other hand, there was observed among these rabbits the unusual occurrence of regression of metastases in superficial parts of the body. The tumors of the iris in 2 rabbits healed during the period of observation and in a 3rd animal they were entirely necrotic at postmortem examination.

Continuous exposure to constant illumination of mercury arcs in crown glass for 3 to 6 months did not appear to induce any outspoken deleterious effect upon the general state of health. All rabbits were given a superabundance of food and those in the light room generally ate more than those in the dark or control rooms, particularly during the first weeks of the experiment. The evidence furnished by body weight determinations, however, indicates that conditions of constant darkness were less favorable than those of constant illumination or of variable diffused sunlight. The changes in mean body weights expressed in percentage values of the initial weights are shown in Table II. The first figures for each experiment include all the rabbits in each group while the second values represent only those animals which survived the observation period of 2 months. On the basis of both computations, the gains in body weight of the groups in the light room were approximately the same or exceeded those in the control room while in only 1 experiment, III, was this the case with the rabbits under conditions of darkness, and here the greater gain

of the surviving rabbits was not as much as that of the surviving animals from the light room. In the other 3 experiments, the groups from the dark room gained much less weight than the controls.

These observations are in general agreement with those obtained in previous experiments in which normal rabbits (6) and rabbits inoculated with *Treponema pallidum* (7) or with the tumor (1) were exposed to conditions of constant light or of constant darkness.

Mortality.—The mortality rates of the control groups ranged from 36.4 per cent in Experiment I to 0 per cent in Experiment II as is seen

TABLE II.

Mean Body Weight. Percentage Variations.

		Controls	Lights	Darks
		per cent	*per cent*	*per cent*
I	All rabbits	+27.9	+24.5	+11.1
	Survivors	+25.9	+24.1	+22.5
II	All rabbits	+10.6	+12.6	+ 2.2
	Survivors	+10.6	+12.6	+ 6.8
III	All rabbits	+22.4	+24.8	+23.0
	Survivors	+20.9	+34.9	+26.4
IV	All rabbits	+33.5	+39.0	+23.2
	Survivors	+35.4	+39.0	+17.3

in Table I. The groups from the dark room showed less variations in this respect, that is from 25.0 to 11.1 per cent, and, as will be recalled, this was also the case in regard to the development of metastases in superficial parts of the body. In 2 experiments, the rates were lower than those of the corresponding control groups (I and III), in another it was the same (IV), while in the last, it was slightly higher (II).

The mortality rates of the light groups also varied considerably, from 30.0 to 0 per cent, but the nature of this variation differed from that of the controls in that there were no deaths in 2 of the groups from the light room while the rates for the other groups were 8.3 and 30.0 per cent as compared with 36.4 and 27.3 per cent for the controls.

The combined mortality rates for the 4 experiments were as follows:

	No. of rabbits	No. of deaths	Mortality rate
			per cent
Controls	37	8	21.6
Darks	38	7	18.4
Lights	36	4	11.1

From these figures as well as from those of individual experiments, it is seen that the highest death rate occurred among the controls and the lowest in the groups kept under conditions of constant illumination while the rate for the groups from the dark room approximated that of the controls.

The great majority of the rabbits in the 4 experiments survived the observation period of 2 months, but from the distribution and character of metastases found at postmortem examination, it is likely that a number would ultimately have died from the effects of the tumor. In some of these animals, a considerable number of metastatic foci were found while in others, there was comparatively little tumor but this involved such structures as both suprarenal glands or the bones of the jaw or the cranial sinuses and adjacent bones. The number of so called probable deaths was lowest in the controls and highest among the dark groups as shown by the following figures:

	No. of rabbits	No. of actual deaths	No. of probable deaths	Incidence of probable deaths	
				Relative rate	Actual rate
				per cent	*per cent*
Controls	37	8	2	5.4	6.9
Darks	38	7	9	23.7	29.0
Lights	36	4	4	11.1	12.5

The actual death rate was, as shown above, slightly higher in the control than in the dark room groups, but if the number of probable deaths is also considered, then the highest total mortality occurred in the latter groups. The lowest rates from both standpoints occurred in the groups exposed to constant light.

The character of the disease in the instances of probable deaths will be discussed later in connection with the distribution of metastases, but it may be said now that the outstanding feature of these cases was the high incidence and number of secondary growths in the bones. This is of special interest in connection with the proportion of actual deaths directly caused by bone involvement and here again the dark group showed by far the highest incidence.

	Actual deaths	Fatal metastases to bone		
		No.	Rate	Location
			per cent	
Controls	8	3	37.8	All spine
Darks	7	5	71.4	4 spine 1 jaw
Lights	4	2	50.0	All spine

Postmortem Observations of Metastatic Foci.—Perhaps the most indicative single feature of the tumor process for evaluating the general character of the disease is the distribution and state of secondary growths

The metastatic foci found at postmortem examination have been analyzed, first, from the point of view of their incidence and total number and, second, from their distribution to certain organs and tissues. As is shown in Table III, the incidence of secondary growths was practically the same in the control and dark groups of 2 experiments, I and II, but it was considerably lower in the case of the light group, while in the 3rd and 4th experiments, it was practically the same in all 3 groups. If the 4 experiments be combined, it is seen from the following figures that there was little difference in the metastatic incidence for the controls and the rabbits from the dark room, but that the rate for the light groups was considerably smaller.

	No. of rabbits	No. with metastases	Incidence
			per cent
Controls	37	21	56.75
Darks	38	23	60.52
Lights	36	14	38.88

The total number of sites involved by secondary growths in the control groups varied greatly, that is, from 6 to 74, and there was much the same variation among the dark groups, that is 13 to 69. In 2 experiments, I and IV, there were slightly fewer foci among the groups from the dark room than among the controls, in No. III, there was the same number, but in the 2nd experiment, there were more in the dark group due to a single instance of a comparatively severe

TABLE III.

Analysis of Metastatic Foci as Observed at Postmortem Examination.

Experiment No.	Group	No. of animals	Incidence		Total No.	Rate		No. of foci in		
			No.	Per cent		Relative	Actual	Deaths	Survivors	
I	C.	11	9	81.9	74	6.7	8.2	26, 19, 14, 6*	4, 2, 1, 1, 1	0 in 2 rabbits.
	D.	12	9	75.0	69	5.8	7.7	19, 6, 2,	24, 6, 5, 5, 1, 1	0 " 3 "
	L.	12	4	33.3	37	3.1	9.3	4	18, 13, 2	0 " 8 "
II	C.	10	3	30.0	6	0.6	2.0	——	3, 2, 1	0 " 7 "
	D.	9	3	33.3	13	1.4	4.3	10	2, 1	0 " 6 "
	L.	10	1	10.0	3	0.3	3.0	——	3	0 " 9 "
III	C.	11	7	63.6	58	5.3	8.3	24, 15, 14	2, 1, 1, 1	0 " 4 "
	D.	12	9	75.0	58	4.8	6.4	12, 6	19, 7, 5, 4, 2, 2, 1	0 " 3 "
	L.	10	7	70.0	44	4.4	6.3	20, 9, 7	3, 3, 1, 1	0 " 3 "
IV	C.	5	2	40.0	34	6.8	17.0	28	6	0 " 3 "
	D.	5	2	40.0	15	3.0	7.5	13	2	0 " 3 "
	L.	4	2	50.0	8	2.0	4.0	——	7, 1	0 " 2 "

* Complicating empyema.

disease in an entire series of very low malignancy. This case was probably a purely chance occurrence which might have been included in either of the other groups, but it is perhaps significant that it did not occur in the light group. The variation in the numbers of metastatic foci was not as marked in the light groups—3 to 44, and there were fewer foci in all light groups than in the corresponding controls. This difference amounted to 50.0 per cent in Experiments I and II, to 24.0 per cent in No. III and to 76.0 per cent in No. IV, in terms of the control values.

The relative rate of metastases, that is the number of foci per rabbit, was uniformly the lowest in the case of all light groups while the rates for the dark groups were somewhat lower than those of the controls except in Experiment II. The value of the relative as contrasted with the actual rate in which only the animals with secondary growths are considered, lies in the fact that the latter is accentuated by the inclusion of 1 or 2 rabbits with numerous metastases. Both rates, however, are useful in furnishing information on the extent of variation in tumor distribution. In these experiments, the control groups were characterized by the most irregular distribution while the groups from the dark room were the most uniform, as shown by the following figures:

Metastatic foci		Controls	Darks	Lights
Relative rate	I.....	6.7	5.8	3.1
	II.....	0.6	1.4	0.3
	III.....	5.3	4.8	4.4
	IV.....	6.8	3.0	2.0
Actual rate	I.....	8.2	7.7	9.3
	II.....	2.0	4.3	3.0
	III.....	8.3	6.4	6.3
	IV.....	17.0	7.5	4.0

If the numbers of foci in the different experiments be combined, the greatest number together with the highest relative and actual rates is found to have occurred in the controls.

	Total No. of foci	Relative rate	Actual rate
Controls.....	172	4.65	8.18
Darks.....	155	3.89	6.43
Lights.....	92	2.55	6.56

As compared with the controls, the distribution of secondary growths was somewhat more restricted among the rabbits from the dark room and markedly so in the animals exposed to constant illumination. On the other hand, it would appear from these combined values that as far as the actual rate is concerned, tumor dis-

tribution was as well marked in the light as in the dark series, a fact which alone might suggest comparable degrees of disease severity. That such was not the case has already been shown by such indices as the mortality rate, the incidence of metastases and the total number of metastatic foci, and an additional demonstration is afforded by an analysis of the character of metastatic foci from the standpoint of the organs and tissues involved.

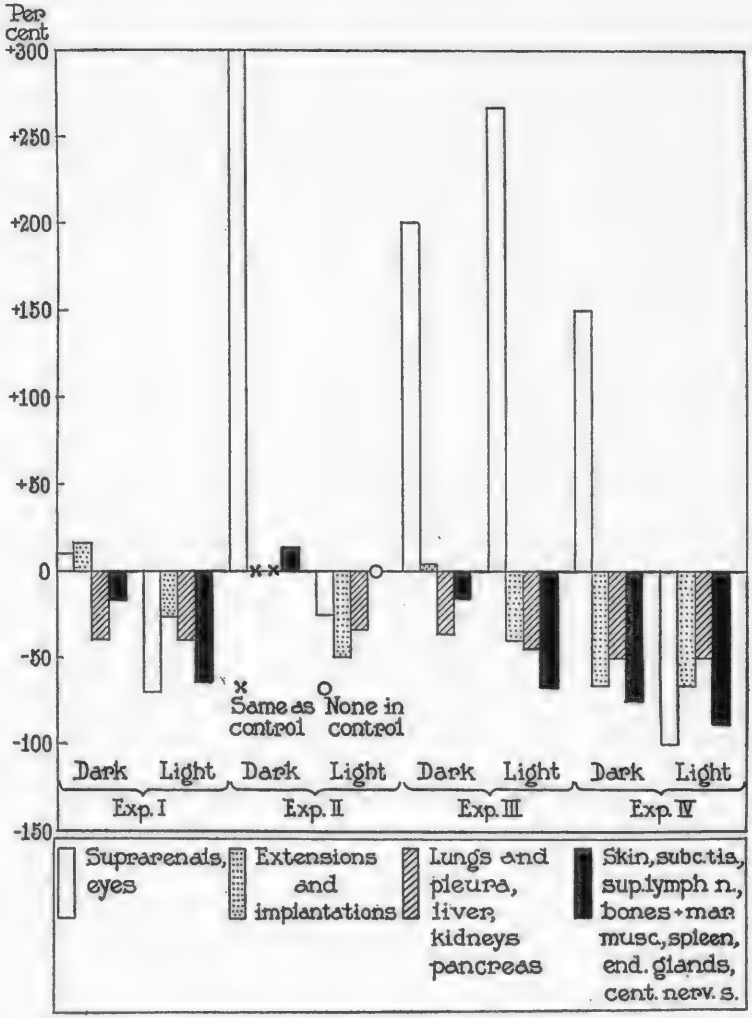
TABLE IV.

Summation Values of Metastatic Foci.

Experiment No.	Group	Suprarenals and eyes	Extensions and implantations	Lungs and pleura, liver, kidneys, pancreas	Skin, subcutaneous tissue, superficial lymph nodes, bones, muscles, endocrine glands, spleen, central nervous system
		per cent	*per cent*	*per cent*	*per cent*
I	C.	250.0	121.2	200.0	103.1
	D.	275.0	136.7	120.0	86.3
	L.	75.0	89.5	120.0	36.7
II	C.	25.0	10.6	60.0	0
	D.	100.0	10.6	60.0	13.2
	L.	0	5.3	40.0	0
III	C.	75.0	105.3	220.0	82.0
	D.	225.0	110.6	140.0	69.8
	L.	275.0	63.3	120.0	26.7
IV	C.	50.0	63.1	80.0	53.3
	D.	125.0	21.1	40.0	13.3
	L.	0	21.1	40.0	6.7

Character of Metastic Involvement.—The method used in determining the relative severity of the disease from the particular organs and tissues involved has already been described in the section on Materials and Methods.

In Text-fig. 1, the summation values of metastatic foci of the dark and light groups are contrasted against those of the controls (Table IV). The most conspicuous feature of this chart are the 4 columns above the base line, representing the greater distribution of metastases to the suprarenals and eyes in 3 dark and 1 light group (Experiments



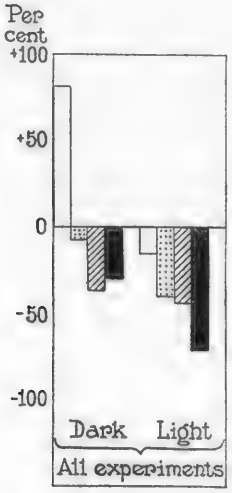
TEXT-FIG. 1. Summation values of metastatic foci in individual experiments expressed in percentage variations of control figures.

II, III and IV). The disproportionate length of these columns as compared with the others is due, first, to the fact that there are only 4 possible sites for secondary growths in this division as contrasted with 5, 19 and 30 in the others, and, second, to the relative frequency of metastases to these organs in cases of low as well as of high malignancy. In so far as severity of the disease is concerned, however, secondary growths in the suprarenals and eyes are of relative unimportance as compared with those to the viscera, other glands of internal secretion, muscles, bones and skin, which in this chart are represented by the last 2 of each set of 4 columns. In the case of the rabbits exposed to constant light, the distribution of metastases to these significant sites of possible tumor growths was, in each experiment, considerably less marked than it was with the controls as indicated by the length of the columns *below* the base line. A further indication of the comparatively limited distribution of metastases in the light groups is brought out by the position of the first 2 columns representing extensions and implantations to the deep lymph nodes and serous membranes and growths in the suprarenals and eyes. These columns are in all but the one instance mentioned above (Experiment III) *below* the base line.

As far as the groups from the dark room are concerned, reference to the chart shows that in 3 experiments, Nos. I, III and IV, metastases to the viscera, endocrine glands, muscles, bones and skin were not as widely distributed as they were in the control groups, but that the difference was not as marked as it was in the case of the rabbits from the light room. In Experiment II, the distribution of tumor in the divisions of extensions and implantations and of the viscera was the same as in the controls, but was slightly greater in the case of the division of skin, bones, etc. This exception, as has already been mentioned, is due to the occurrence of 1 instance of a comparatively severe disease in a series of low malignancy.

The last chart (Text-fig. 2) in which the summation values for metastatic foci of the 4 experiments have been combined illustrates the outstanding features of the distribution of secondary growths in the rabbits exposed to constant illumination or to constant darkness, as compared with those kept under indoor conditions of variable diffused sunlight. In the case of the animals from the light room,

there was a much more limited distribution of tumor in all 4 divisions of metastatic foci, especially in the last division which is particularly involved in instances of pronounced malignancy. In the case of the rabbits from the dark room, secondary growths were also less widely distributed to 3 divisions of possible tumor foci, but were more frequent in the division of the suprarenals and eyes. As compared with the light groups, the reduction of summation values was not as pronounced.



TEXT-FIG. 2. Combined summation values of metastatic foci expressed in percentage variations of control figures.

In discussing the mortality rate, mention was made of the probability that if certain rabbits had been allowed to live beyond the experimental period of 2 months, they would ultimately have died from the effects of tumor growth and statistics were given which showed that the largest number of these cases was found in the groups from the dark room. This aspect of the disease deserves further discussion and may conveniently be considered at this point in connection with the general character of metastatic distribution.

There were 4 instances equally divided between the dark and light

groups of relatively malignant but slowly progressive tumors (Table III). Postmortem examination 2 months after inoculation revealed numerous foci of secondary growth which would certainly have brought about a fatal termination had the animals been allowed to live beyond the experimental period. There were no control rabbits with this type of disease. No difficulty is experienced in recognizing such cases of probable deaths, but there were 11 other rabbits which have been similarly classed because of the location and state of the metastases, although these were comparatively few in number. There were 7 such instances in the dark, 2 in the control and 2 in the light groups, respectively.

The most frequent sites of secondary growths in this type of presumably fatal disease are the suprarenal glands, but in these experiments, there was also an unusual number of metastases to the bones of the face and jaws and the cranial sinuses, particularly in the rabbits from the dark room as shown by the following combined group figures:

	Probable deaths omitting malignant cases	Total foci of metastases	Foci of bone metastases
Darks	7	27	6 or 22.2 per cent
Lights	2	10	1 " 10.0 " "
Controls	2	9	0 " 0 " "

These findings are in line with the greater incidence of bone metastases in the dark groups than in either the control or light series. The observations of individual experiments, as well as the combined values are given below to show that this condition was present in each experiment.

Incidence of Metastases to Bone.

	Controls	Darks	Lights
	No. of rabbits	No. of rabbits	No. of rabbits
Experiment I	2 or 18.2 per cent	7 or 58.3 per cent	2 or 16.7 per cent
" II	0 ——	1 " 11.1 " "	0 ——
" III	3 " 27.3 " "	5 " 41.7 " "	3 " 30.0 " "
" IV	1 " 20.0 " "	2 " 40.0 " "	1 " 25.0 " "
Total	6 " 16.2 " "	15 " 39.4 " "	6 " 16.7 " "

It will be recalled that in discussing the general character of the disease in the control groups, it was pointed out that the incidence of bone lesions was considerably greater than in the first 20 generations of the tumor. The above data show that in respect to the incidence of this type of metastasis the groups from the light room did not differ from the controls, but that there was a much higher rate among the dark groups. The environment of constant darkness evidently favored the increased tendency toward bone lesions prevailing at this time.

The foregoing analysis of clinical and postmortem data may be briefly summarized at this point before discussing the significance of the results obtained. The tumor process in the control groups exposed to variable diffused sunlight varied from a very mild condition in 1 experiment to a considerably more severe disease in 2 others, but in none of the 4 was a level of well marked malignancy attained. It was much more severe, however, than the disease which developed in the groups exposed to constant illumination and somewhat more so than in the groups kept in constant darkness. As compared with the controls the light groups were characterized by the following features:—an unusual number of failures to obtain a primary growth, less active and less extensive primary tumors, a lower mortality rate, a lower incidence and a smaller number of metastases recognizable during life and at postmortem examination and a much greater restriction in the distribution of these growths. It is significant, moreover, that the influence of constant illumination in the direction of diminished malignancy was observed at times of mild as well as of more severe disease.

A similar comparison of dark groups showed that the growth of the primary tumors was more irregular but on the whole not as pronounced or as persistent as in the controls. On the other hand, the mortality rates and the incidence of metastases found during life and at autopsy were only slightly lower than control values. The number of metastases, however, was smaller, the relative and actual rates were lower and the distribution of these growths was definitely more restricted. An interesting aspect of the tumor process in these rabbits was the tendency toward group uniformity in regard to certain features of the disease. Thus, in the 4 experiments the dark groups as compared

with the control or the light series, showed less variation in the incidence of secondary growths recognizable during life, in the mortality rates and in the relative and actual rates of metastatic distribution. A similar tendency has also been observed in the dark groups of experiments with *Treponema pallidum* (7). Although the influence of constant darkness was exerted toward diminishing tumor malignancy, it was much less pronounced than that of constant illumination, and, furthermore, it had little or no effect in the series in which the disease was of a mild character. It was also modified by the peculiar nature of the tumor process of the 4 experiments, which was characterized by a high incidence of bone metastases despite the moderate or low level of malignancy that prevailed. Conditions of constant darkness were associated with a greatly increased incidence of these lesions which, due to their location in such sites as the spinal column and jaws, were directly responsible for a large proportion of actual as well as of probable deaths.

The results of a previous experiment (1) with constant illumination (Mazda lamps and mercury arcs in crown glass) and constant darkness were of the same order of diminished malignancy but more striking. This was especially true of the group from the light room and while the difference in the case of the dark group was not pronounced, it was definitely greater than in the current experiments. The present analysis would tend to show that the greater severity of the tumor process prevailing at the time of the previous experiment undoubtedly was 1 factor that influenced the particular character of the results obtained. The 2 types of illumination must also be considered, and from this standpoint the combination of Mazda lamps with mercury arcs was more effective than the arcs alone, if one may judge from the single experiment in which the former system was used.

It is obvious that before one can ascribe the effect of diminished malignancy solely to the influence of constant illumination or of constant darkness, other possible factors must be considered. The state or condition of the animal organism prior to exposure to these surroundings is probably of considerable significance and it is conceivable that different states of the host might react in a different manner to these conditions of light and darkness with materially

dissimilar results. Indeed, our explanation of the variations in tumor malignancy is largely based upon the idea that resistant or susceptible states of the host are dependent upon different conditions of animal economy, which may be affected by innumerable factors.

One must also take into account the fact that in these experiments, the animals were subjected to the influence of the factor of change incident to their removal from the variable diffused sunlight of the laboratory to constant conditions of continuous light or of continuous darkness. Little is known of the importance of this factor in relation to the reaction of animals to experimentally induced disease but there are certain observations with this tumor which suggest that the effect of change in external conditions may manifest itself in the direction of increased malignancy. For instance, in 1 experiment dealing with this question, the tumor process in a group of 10 rabbits recently brought to the laboratory was much more severe than in a similar sized group assembled 2 months prior to inoculation.

In order, therefore, that the influence of such a physical force as constant illumination on tumor malignancy be fully effective, it is probable that some sort of initial adjustment or accommodation to the new environment must take place, as experiments begun before its completion would necessarily be complicated by the process whatever it may be. The length of time required for such an adjustment under the conditions of these experiments is not known, but previous work dealing with the changes in organ weights of normal rabbits under similar environmental surroundings, suggested that a 4 weeks' period was associated with relatively stable weights of most organs (6). Consequently, in the majority of tumor experiments, inoculations have been carried out after 4 weeks' exposure to the light or dark conditions. In this connection, the results of an inoculation after 2 weeks' exposure are of interest. The group from the light room showed a less severe disease than the controls, but in the group from the dark room, the tumor process was distinctly more malignant. While the observations of a single experiment are not conclusive, the results of the dark group are in line with what might be expected if the 2 weeks period was not sufficient for the completion of adjustment to this condition. Under these circumstances, the effect of the factor of change would still be operative and if its influence in the

direction of increased malignancy were sufficient, one would expect a more severe disease. On the other hand, a 2 weeks' period was evidently sufficient as far as constant illumination was concerned for the establishment of an adjustment of the animal organism to this condition, or, and this may prove to be the correct explanation, the effect of the light might have been such as to mask or effectually modify the influence of other factors operating in the direction of increased malignancy.

The results of these experiments are interpreted as furnishing evidence in support of the belief referred to in the beginning of this paper, that there is a relation between the factor of light and the manifestations and course of the disease induced by a transplantable malignant neoplasm. The mechanism by which the effects are produced is not known. We have considered the influence of the seasonal factor in this condition and in experimental infections of the rabbit induced by *Treponema pallidum* as operating upon or affecting animal economy and susceptibility or resistance as a functional activity of the animal organism. From this standpoint, it appears that resistance to tumor growth as displayed by rabbits living under certain conditions of constant illumination and to a less extent, in almost constant darkness, was more pronounced than it was in the case of control rabbits exposed to the daily fluctuations of diffused sunlight.

SUMMARY.

Experiments are reported in which an environment of constant and continuous light with a wave-length of from 3022 to 5790 Ångström units, supplied by mercury arcs in crown glass, and of constant darkness, have influenced the course and character of a malignant disease of rabbits induced by a transplantable neoplasm.

Under the influence of constant light the level of malignancy was lower than in control animals living under ordinary conditions of diffused sunlight. Under the influence of constant darkness the level of malignancy was somewhat lower than in control animals but the effect of this environment was modified by the special character of the disease prevailing at this time. The incidence of bone metastases was unusually high, but it was greatly increased in the rabbits kept in constant darkness.

These observations furnish experimental evidence in support of the belief that there is a correlation between the external factor of light and the manifestations of an experimental malignant disease.

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